Re-design of *Rhodobacter sphaeroides* Dimethyl Sulfoxide Reductase

ENHANCEMENT OF ADENOSINE N³-OXIDE REDUCTASE ACTIVITY*

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The periplasmic DMSO reductase from *Rhodobacter sphaeroides* f. *denitrificans* has been expressed in *Escherichia coli* BL21(DE3) cells in its mature form and with the *R. sphaeroides* or *E. coli* N-terminal signal sequence. Whereas the *R. sphaeroides* signal sequence prevents formation of active enzyme, addition of a 6× His-tag at the N terminus of the mature peptide maximizes production of active enzyme and allows for affinity purification. The recombinant protein contains 1.7–1.9 guanines and greater than 0.7 molybdenum atoms per molecule and has a DMSO reductase activity of 3.4–3.7 units/nmol molybdenum, compared with 3.7 units/nmol molybdenum for enzyme purified from *R. sphaeroides*. The recombinant enzyme differs from the native enzyme in its color and spectrum but is indistinguishable from the native protein after redox cycling with reduced methyl viologen and Me₂SO. Substitution of Cys for the molybdenum-ligating Ser-147 produced a protein with DMSO reductase activity of 1.4–1.5 units/nmol molybdenum. The mutant protein differs from wild type in its color and absorption spectrum in both the oxidized and reduced states. This substitute leads to losses of 61–99% of activity toward five substrates, but the adenosine N³-oxide reductase activity increases by over 400%.

The majority of molybdoenzymes contain one or more strongly chromophoric prosthetic groups in addition to molybdenum (1, 2). Although the properties of their molybdenum centers have been studied by EPR and EXAFS (1), other techniques such as MCD, resonance Raman spectroscopy (RR), and UV-visible absorption spectroscopy have not been applicable to these enzymes because the weakly absorbing molybdenum centers are masked by the other stronger chromophores. Periplasmic *Rhodobacter* DMSO reductase is one among the few proteins containing only the molybdenum cofactor as a prosthetic group (3). The active enzyme is also a monomer of 85 kDa in contrast to other well characterized molybdoenzymes that are larger oligomeric molecules, many containing a membrane-anchoring subunit complicating molecular cloning and purification (4). All of these factors combine to make *Rhodobacter* DMSO reductase an ideal molybdoenzyme for cloning and structural studies of the molybdenum active site.

The purified *Rhodobacter sphaeroides* enzyme has been studied by several spectroscopic techniques including absorption spectroscopy (3), EPR (3, 5), VTMCD (5), EXAFS (6), and RR (7). EXAFS studies of the oxidized *R. sphaeroides* protein showed the molybdenum ligand field to contain 4 Mo–S, 1 Mo=O, and 1 Mo–O bonds (6), and chemical purification of the purified enzyme revealed the bis(MGD) molybdenum form of the cofactor (Fig. 1A) (8). The long wavelength absorption bands in the visible spectrum of DMSO reductase have proven to be extremely useful for RR, enabling detailed and nearly complete assignments for all of the observed RR resonance lines. In particular, redox cycling of the enzyme in the presence of ¹⁸O-labeled Me₂SO caused a downshift of 43 cm⁻¹ in the bond assigned to Mo=O, providing unequivocal evidence for the single oxo group. This finding also demonstrated the mono-oxo molybdenum center is fully capable of carrying out an oxo transfer reaction (7).

As mentioned above, EXAFS studies on *R. sphaeroides* DMSO reductase indicated that the ligand field of molybdenum includes an Mo–O bond presumably from a protein side chain (6). Subsequent x-ray crystallography of the enzyme identified this protein ligand as Ser-147 (9). Amino acid sequence alignment of the DMSO reductase family of proteins suggests that the protein ligand to molybdenum in diverse members of the group may be either Ser, Cys, or selenocysteine (Fig. 1B) (9) with the latter residue identified as the molybdenum ligand in *Escherichia coli* formate dehydrogenase H (10, 11). The reason for this diversity in protein molybdenum ligation is not known.

An exciting recent development in the molybdoenzyme field is the elucidation of the x-ray crystallographic structures of a number of proteins (9, 11–15), including at least one from each of the three major families, setting the stage for detailed structure-function studies relating to substrate specificities, identification of active site residues, and delineation of internal electron transfer pathways in proteins containing multiple prosthetic groups. In particular, the effect of site-directed mutagenesis on the electronic and chemical properties of the molybdenum centers can be examined using kinetic techniques, analysis of substrate specificities, and a variety of spectroscopic techniques including EXAFS, RR, EPR, and x-ray crystallography.

In order to carry out detailed structural and mechanistic studies on *R. sphaeroides* DMSO reductase, we have cloned the *R. sphaeroides* DMSO reductase gene (16) with the intent of expressing it heterologously in *E. coli* and generating site-directed mutants. Although other laboratories have also reported the cloning of the *R. sphaeroides* (17) and *Rhodobacter capsulatus* (18, 19) DMSO reductase structural genes, no successful heterologous expression and purification of the active enzyme has been reported. Knablein et al. (20) have homologously expressed the cloned DMSO reductase structural gene.
in *R. capsulatus* in an active form, although the enzyme has not been purified or characterized. Since plasmids cannot be introduced into *Rhodobacter* by transformation, homologous expression requires transformation of a donor *E. coli* strain with a suicide plasmid followed by conjugation and subsequent crossover to integrate a mutated gene into the *R. capsulatus* cellular chromosome. These complications are eliminated by heterologous expression in *E. coli*, with the additional advantage of a multitude of molecular biology techniques tailored to this system. Furthermore, because of the availability of *E. coli* mutants defective in cofactor biosynthesis (21), heterologous expression provides the ability to examine the mechanism of incorporation of the complex cofactor into the apoprotein.

Initial attempts at heterologous expression of the cloned *R. sphaeroides* DMSO reductase gene in *E. coli* produced only inactive protein (16). Since other molybdoenzymes including the human and rat sulfite oxidases (22, 23) and *E. coli* DMSO reductase (24) have been successfully cloned and expressed in *E. coli*, the apparent lack of expression of active *R. sphaeroides* DMSO reductase was unexpected. The cloned *R. sphaeroides* DMSO reductase gene codes for a precursor protein that includes an N-terminal 42-residue signal sequence serving to target the mature protein to the periplasmic space. *R. sphaeroides* DMSO reductase shares approximately 50% amino acid sequence identity with mature *E. coli* TMAO reductase, another periplasmic molybdoenzyme (25). Although the mature enzymes share significant homology, the periplasmic signal sequences differ substantially (26). Since differences in the signal sequence requirements of the two organisms could result in improper processing, we have examined the effects of deletion of the N-terminal signal sequence as well as the effects of substitution of the *R. sphaeroides* DMSO reductase signal sequence with that of *E. coli* TMAO reductase on the expression of active DMSO reductase. These studies show that maximum expression of active enzyme is achieved without any signal sequence. Paradoxically, the inclusion of an N-terminal His-tag results in an even higher production of active enzyme and facilitates rapid and high yield purification of the recombinant protein.

To explore the effect of ligand substitution on catalytic activity and electrochemical properties such as oxidation-reduction potentials, we have created the S147C mutant of DMSO reductase and examined its properties. As described below, the mutant enzyme displays attenuated activities with several substrates but markedly elevated activity with adenosine N-oxide. These data as well as the results of spectroscopic analysis of the recombinant wild type and the S147C forms of DMSO reductase are presented in this paper.

**EXPERIMENTAL PROCEDURES**

**Materials**—T4 polynucleotide kinase, T4 DNA ligase, calf intestinal alkaline phosphatase, 1-kilobase pair DNA ladder standards, Miller’s LB broth, and competent DH5α and DMI *E. coli* cells were from Life Technologies, Inc. XL1-Blue *E. coli* cells and pBluescript II KS(+) were from Stratagene. Restriction enzymes were from Stratagene and Life Technologies, Inc. AmpliTaq DNA polymerase was from Perkin-Elmer. Glycereol-tolerant DNA sequencing gel mix was from Amersham Pharmacia Biotech. The Transformer Site-directed Mutagenesis Kit and BMH 71–18 mutS cells were from CLONTECH. The pET-28 and pET-29 expression systems, BL21(DE3) *E. coli* cells, induction control plasmid F, pLysE, and Perfect Protein Standards were from Novagen. The TA cloning kit, including the pCRII vector and INVαF *E. coli* cells, was from Invitrogen. BCA protein assay reagents and 1-step chloronaphthol were from Pierce. Ultrafiltration devices and membranes were from Millipore. Q-Sepharose Fast Flow, Sephadex G-25, and Superox 12 resins were from Amersham Pharmacia Biotech. Ni-NTA agarose resin was from Qiagen. Electrophoresis reagents, protein mini-gels, prestained low range SDS-polyacrylamide gel electrophoresis standards, and gelatin were from Bio-Rad. Goat anti-rabbit horseradish peroxidase-conjugated IgG was from Boehringer Mannheim. The atomic absorption molybdenum standard was from J. T. Baker Inc. All other reagents were obtained from Sigma with the exceptions of IPTG, obtained from Research Products International, Me2SO, obtained from Mallinckrodt, and D-biotin D-sulfoxide, prepared as described by Pollock and Barber (27).

**Recombinant DMSO Reductase Expression Constructs**—Oligonucleotides were synthesized at the Duke University DNA Core Facility on an Applied Biosystems DNA synthesizer model 394. Site-directed mutagenesis of *R. sphaeroides* DMSO reductase with the CLONTECH Transformer Site-directed Mutagenesis Kit was carried out on double-stranded DNA according to the manufacturer’s protocol. The pJH115 construct (Table I), containing the DMSO reductase coding sequence, free of NcoI and NdeI restriction sites, in pBluescript II KS(+), was subjected to mutagenesis with selection primer BSXBaD677 (Table II) and mutagenic primers NdeA122 or NdeA-2 to create pJH115 and pJH119, respectively. The NdeI restriction site created at position 122 in pJH115 added a Met codon to the first position of the mature enzyme.
TABLE I

<table>
<thead>
<tr>
<th>Construct</th>
<th>Vector Insert</th>
<th>5′-Cloning site</th>
<th>3′-Cloning site</th>
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<tr>
<td>pJH115</td>
<td>pBluescript II KS(+)</td>
<td>2.7 kb containing complete CDS with NcoI sites at positions 726</td>
<td>PstI</td>
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<tr>
<td>pJH118</td>
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<td>PstI</td>
</tr>
<tr>
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<td>pJH115 insert with NdeI site created at position –2</td>
<td>PstI</td>
</tr>
<tr>
<td>pJH130</td>
<td>pBluescript II KS(+)</td>
<td>pJH115 insert with BclI site created at position 123</td>
<td>PstI</td>
</tr>
<tr>
<td>pHE520</td>
<td>pET-29</td>
<td>Mature CDS from pJH118 in frame with vector initiation codon (no S-Tag)</td>
<td>NdeI</td>
</tr>
<tr>
<td>pHE521</td>
<td>pET-29</td>
<td>Precursor CDS from pJH119 in frame with vector initiation codon (no S-Tag)</td>
<td>NdeI</td>
</tr>
<tr>
<td>pTT601</td>
<td>pCRII</td>
<td>torA signal sequence</td>
<td>NdeI</td>
</tr>
<tr>
<td>pHE527</td>
<td>pET-29</td>
<td>torA signal sequence and mature DMSO reductase CDS in frame with vector initiation codon (no S-Tag)</td>
<td>NdeI</td>
</tr>
<tr>
<td>pJH720</td>
<td>pET-28</td>
<td>Mature CDS from pJH118 in frame with N-terminal 6× His-tag</td>
<td>NdeI</td>
</tr>
<tr>
<td>pJH731</td>
<td>pET-28</td>
<td>Mature CDS from pJH130 in frame with N-terminal 6× His-tag</td>
<td>NdeI</td>
</tr>
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</table>

a kb, kilobase pairs.

TABLE II

<table>
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<tr>
<th>Name</th>
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<th>Positionb</th>
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<tr>
<td>NdeA-2</td>
<td>aagaaggaagcaatgactagaaggtgtc</td>
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<tr>
<td>NdeA2</td>
<td>ggcggcggcgeatcgcaggtctgcge</td>
<td>109 to 138</td>
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<tr>
<td>TRNdeA-2</td>
<td>gagagggggcgeactaaggtttc</td>
<td>torA 12 to 18</td>
</tr>
<tr>
<td>TBBclA122</td>
<td>ctctggggagactacaagtggttcag</td>
<td>torA 118 to 150</td>
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<tr>
<td>BclA123</td>
<td>gcggcggcggcgeatcgcaggtctgcge</td>
<td>109–140</td>
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<tr>
<td>S147C</td>
<td>cggcgccgctggagactacaagtggttcag</td>
<td>549–582</td>
</tr>
<tr>
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<td>pBluescript II KS 688–697</td>
</tr>
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<td>ggcccgccgctggagactacaagtggttcag</td>
<td>pBluescript II KS 688–697</td>
</tr>
</tbody>
</table>

a Mismatches are indicated in bold.
b Nucleotide position in DMSO reductase coding sequence unless otherwise specified.

coding sequence, whereas the coding sequence of the precursor enzyme in pJH119 remained unaltered. The inserts were released with NdeI and HindIII and ligated into the pET-29 expression vector with the initiation codons for the mature or precursor peptides positioned at the translation start site of the vector, creating pJH520 and pJH521, respectively. Similar ligation of the insert from pJH119 into the pET-28 expression vector positioned the mature coding sequence in frame with the 6× His-tag coding sequence present in the vector, creating pJH720.

Creation of the DMSO Reductase/TMAO Reductase Fusion Construct—PCR primers TRNdeA-2 and TRBclA132 were designed to amplify the first 150 nucleotides of the torA coding sequence from E. coli DH5α genomic DNA, incorporating an NdeI site at position –2 and a BclI site at position 132, respectively. The 100-μl PCR reaction contained 0.8 μg of DNA, 4 mM MgCl₂, 1× Taq PCR buffer II, 20 mM of dNTP, 50 pmol of each primer, and 2.5 units of AmpliTaq DNA polymerase. The PCR parameters included an initial 2-min 94 °C denaturation, 15 amplification cycles (30 s denaturation at 97 °C, 30 s annealing at 54 °C, 1-min extension at 74 °C), and a final 10-min 72 °C extension. The PCR product was ligated directly into the pCRII vector using the TA cloning kit, and the construct, pTT601, containing the insert, was isolated from E. coli InvA™ cells that had been transformed with the ligated material. Mutagenesis with selection primer BSXbaD677 and mutagenic primer BclA123 on pJH115 incorporated a BclI site at position 123 of the gene creating pJH125. Unmethyled pJH125 and pTT601 constructs were propagated in dam−/dcm− DM1 cells, and the torA insert was released from pTT601 with SpeI and BclI and ligated into pJH125 that had been digested with the same enzymes, creating the fusion construct pJH126. The coding sequence for the fusion protein was released from pJH126 with NdeI and HindIII and ligated into the pET-29 expression vector with the initiation codon of the insert positioned at the translation start site of the vector, creating pJH527.

Preparation of S147C Constructs—Construct pJH118 was subjected to mutagenesis with selection primer BSXbaD677 and mutagenic primer S147C to alter the Ser codon at position 147 to a Cys codon creating pJH130. The mutant coding sequence for the mature peptide was released from the construct with NdeI and HindIII and ligated into pET-28 in frame with the 6× His-tag coding sequence creating pJH731.

DNA Sequencing—Inserts containing the DMSO reductase coding sequence were sequenced using the chain termination method on double-stranded DNA with the Oncor Fidelity DNA Sequencing System according to the manufacturer’s protocol. Sequencing gels containing 6% acrylamide were prepared from Glycerol-tolerant DNA Sequencing Gel Mix and included 20% formamide to resolve compressions. Sequence analysis was accomplished using the Wisconsin Sequence Analysis Package, Version 8 (Genetics Computer Group, Inc.). Alternatively, automated sequencing was performed at the Duke University DNA Analysis Facility using a Perkin-Elmer Dye Terminator Cycle Sequencing system with AmpliTaq DNA polymerase combined with an Applied Biosystems 377 PRISM DNA Sequencing instrument.
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solution was centrifuged again. The supernatant was combined with 15–25 ml of Ni-NTA resin, and the slurry was equilibrated with gentle stirring at 4 °C for 15 min. The slurry was poured into a 2.5 × 10-cm chromatography column, and the protein solution was allowed to flow through the resin. The column was then washed with 2 column volumes of 100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 300 mM NaCl, pH 8.0. Fractions containing the DMSO reductase were combined and made 20% saturated with solid ammonium sulfate. The protein was loaded on a 23-ml phenyl-Sepharose HR10/50 column equilibrated in 114 g/liter ammonium sulfate in 50 mM Tris-HCl, pH 8.5. The column was then washed with 1 column volume of this solution followed by a 1-column volume gradient that decreased the ammonium sulfate concentration from 114 to 34 g/liter (NH4)2SO4, a 1-column volume wash at 34 g/liter, a 2-column volume gradient further dropping the ammonium sulfate concentration to 17 g/liter, and finally a 4-column volume gradient decreasing the ammonium sulfate concentration from 17 to 0 g/liter. Selected fractions were combined, and the ammonium sulfate was removed by ultrafiltration using Amicon PM30 membranes or Centricon 30 devices.

**Polyacrylamide Gel Electrophoresis and Western Blot Analysis—** Samples were heated at 95 °C in sample buffer containing 2% SDS and 5% 2-Mercaptoethanol. Electrophoresis was carried out on a 4–20% gradient polyacrylamide Ready Gels using the Bio-Rad Mini-PROTEAN II Electrophoresis System at 200 V, and proteins on the gels were stained with Coomassie Blue or transferred to nitrocellulose membranes using the Bio-Rad Mini Trans-Blot electrophoretic transfer cell. Western blot analysis was performed as described previously (16).

**Activity and Protein Assays—** Pure samples of DMSO reductase were quantitated spectrophotometrically at 280 nm using an extinction coefficient of 200,000 M<sup>-1</sup> cm<sup>-1</sup> or 2.3 (mg/ml)<sup>-1</sup> cm<sup>-1</sup> (8). Total protein was also assayed using the Pierce BCA assay on trichloroacetic acid-precipitated samples as described in the manufacturer’s protocol, with known concentrations of DMSO reductase purified from *R. sphaeroides* as standards. DMSO reductase activity was assayed as described previously (3) in a 2-ml anaerobic assay mixture containing up to 3 μg of the enzyme, 0.1 mM sodium dithionite-reduced methyl viologen, 50 mM Tris-HCl, pH 7.5, and 10 mM Me2SO. The assay was initiated by injecting sodium dithionite into a sealed anaerobic mixture containing all other assay components. The change in absorbance at 600 nm was monitored for 1 min, and the activity in micromoles of Me2SO reduced per min was determined by the formula \( \frac{U}{1/2(\Delta A_{600\text{nm}})/\text{min} \times \text{[Me}_2\text{SO]}_0 \times v} \) where \( \Delta A_{600\text{nm}} \) absorbance change at 600 nm, is \( 1.2 \times 10^{-2} \mu\text{M} \times 1 \cdot \text{cm}^{-1} \) and \( v = 2 \times 10^{-3} \text{ liter} \). Alternative substrates were assayed using enzyme concentrations that varied relative to the activity toward that substrate. The substrates TMAO, m-MetSO, potassium chloride, fumaric acid, and potassium nitrate were assayed at 10 mM substrate concentrations, whereas BSO was assayed at 1.7 mM (30). ANO was dissolved directly in the assay buffer at 3.4 mM (31).

**DMSO Reductase Activity Stains—** Solid ammonium sulfate was added to soluble cell extracts to 45% saturation at 4 °C, and the precipitated material was removed by centrifugation at 23,000 × g for 15 min. The supernatant was heated rapidly to 60 °C, and immediately chilled in an ice bath, and the precipitated material was again removed by centrifugation. The ammonium sulfate concentration of the supernatant was increased to 70% saturation, and after centrifugation the supernatant was discarded. The precipitated protein was dissolved in 50 mM Tris-HCl, pH 7.5, and SDS-free samples containing 25–30 μg of total protein were loaded onto 4–20% Ready Gels. After non-denaturing electrophoresis at 200 V for approximately 30 min, the gels were stained in a 13-ml anaerobic solution of 150 mM Me2SO, 4 mM reduced methyl viologen, and 77 mM Tris-HCl, pH 7.5 (30). After bands containing Me2SO reducing activity became clear, the stained background was fixed with 2.5% tetrazolium red (33).

**Absorption Spectroscopy—** Absorption spectroscopy was carried out using a Shimadzu UV-2101 PC spectrophotometer and quartz cuvettes. In order to record the spectrum of purified recombinant DMSO reductase that had been subjected to more catalytic cycles, the protein was added to 0.5 mM DMSO reducing activity in an anaerobic environment until the methyl viologen became dark purple in color. The enzyme was reoxidized by dropwise addition of 2 mM Me2SO. After reoxidation, the purified enzyme was concentrated and passed through an HR16/50 Superose 12 FPLC column to remove the methyl viologen and Me2SO while ensuring homogeneous enzyme samples. Selected fractions containing the DMSO reductase were combined and concentrated to at least 5 mg/ml. To obtain a reduced spectrum of the S147C variant, the enzyme was reduced more active enzyme as revealed by activity-stained native polyacrylamide gels (Fig. 2B). Expression of DMSO reductase with the TMAO reductase signal sequence resulted in the production of active enzyme when expressed at 26 °C (Fig. 2B); however, this fusion protein was not investigated further due to an active enzyme yield that was lower than that of the much simpler mature, recombinant form. The expression results suggest that the 42-residue periplasmic signal sequence found on the precursor enzyme (16, 35) may actually interfere with formation of the active *R. sphaeroides* enzyme in the cytoplasm.
of mature DMSO reductase purified from R. sphaeroides DMSO reductase activity stain of ammonium sulfate and heat-treated contains prestained low range molecular weight standards.

Lane 8 is from cells containing pET-29 with no insert. Fusion protein expressed from pJH527 at 37 and 26 °C, respectively. and the mature enzyme alone. It has been demonstrated that the pressing recombinant DMSO reductase fused with the His-tag with the His-tag encoded by the vector. BL21(DE3) cells expressed the signal sequence was not necessary for the production of active enzyme 2 and that expression of the DMSO reductase was shown to be more effective and anaerobic respiratory enzymes (36, 37). Expression of recombinant E. coli DMSO reductase purified from the BL21(DE3) cells also contained long wavelength bands (Fig. 4), but the absorption peaks are at 380, 470, and 650 nm (Table IV) (3). The spectrum of the green recombinant DMSO reductase purified from the BL21(DE3) cells also contains long wavelength bands (Fig. 4), but the absorption peaks are at 380, 470, and 650 nm (Table IV). Because the recombinant protein was purified from cells that were not dependent upon the reduction of Me2SO for growth and an endogenous reductant of the enzyme may not be present, it appeared that the recombinant enzyme may not have been subjected to catalytic turnover after assembly. Accordingly, the effect of redox cycling on the recombinant enzyme was examined. Complete reduction of the green recombinant DMSO reductase with excess reduced methyl viologen followed by controlled reoxidation by dropwise addition of dilute Me2SO generated a product with an absorption spectrum almost indistinguishable from that of the R. sphaeroides DMSO reductase (Fig. 4).

The substitution of a Cys residue for the molybdenum-ligating Ser-147 was accomplished through site-directed mutagenesis of pJH118. The coding sequence for this variant was ligated into pET-28 to create pJH731, such that the expression product of this construct consisted of the mature peptide containing the substituted Cys at position 147 and a 6× His-tag. The product of this construct consisted of the mature peptide containing the substituted Cys at position 147 and a 6× His-tag at the N terminus. BL21(DE3) cells containing the construct were grown anaerobically at room temperature on M9ZB supplemented with fumarate and glucose. Expression was induced by dropwise addition of dilute Me2SO generated a product with an absorption spectrum almost indistinguishable from that of the R. sphaeroides DMSO reductase (38). Typically, 40 liters of cells grown under these conditions released more than 20 mg of soluble DMSO reductase upon lysis. The cells were harvested by centrifugation, resuspended, and frozen. The cells were lysed by thawing, and the DMSO reductase was purified 130-fold in a three-step purification procedure that includes a 50 °C heat step, metal affinity chromatography using Ni-NTA resin, and hydrophobic interaction chromatography on a phenyl-Sepharose FPLC column (Table III). This procedure results in recovery of 31% of the DMSO reductase activity originally present in the extracts, and the purified enzyme appears as a single band on a Coomassie-stained SDS-polyacrylamide gel (Fig. 3).

Whereas DMSO reductase purified from R. sphaeroides cells grown anaerobically in medium containing Me2SO as the terminal electron acceptor is dark brown in color, the recombinant mature R. sphaeroides DMSO reductase purified from the BL21(DE3) cells is green-colored. As reported previously, the absorption spectrum of the brown DMSO reductase purified from R. sphaeroides cells displays maxima at 355, 470, 550, and 720 nm (Table IV) (3). The spectrum of the green recombinant DMSO reductase purified from the BL21(DE3) cells also contains long wavelength bands (Fig. 4), but the absorption peaks are at 380, 470, and 650 nm (Table IV). Because the recombinant protein was purified from cells that were not dependent upon the reduction of Me2SO for growth and an endogenous reductant of the enzyme may not be present, it appeared that the recombinant enzyme may not have been subjected to catalytic turnover after assembly. Accordingly, the effect of redox cycling on the recombinant enzyme was examined. Complete reduction of the green recombinant DMSO reductase with excess reduced methyl viologen followed by controlled reoxidation by dropwise addition of dilute Me2SO generated a product with an absorption spectrum almost indistinguishable from that of the R. sphaeroides DMSO reductase (Fig. 4).

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**TABLE III**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Recovery</th>
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<td>180</td>
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*Units, μmol of Me2SO consumed per min.*
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The stoichiometry of molybdenum and MGD in the recombinant enzyme is 3.7 units/nmol molybdenum. The specific activity of the recombinant enzyme is 3.4–3.7 units/nmol molybdenum.

The specific activity of the recombinant variant is 3.4–3.7 units/nmol molybdenum (93–100% of wild type) and that of the S147C variant is 1.4–1.5 units/nmol molybdenum (37–41% of wild type).

The specific activity of the S147C mutant was examined with TMAO, MetSO, chlorate, BSO, and ANO (Table VI). The S147C molybdenum ligand substitution results in nearly a total loss of the ability to reduce MetSO, chloride, and BSO as well as a 61% decrease in the ability to reduce TMAO. However, the mutation increases the ANO reducing activity of the enzyme by greater than 400%, exceeding the specific activity of the wild type enzyme toward any substrate measured. Surprisingly, the S147C substitution increases the $K_m$ for Me$_2$SO from the wild type value of 7 $\mu$M to 1 mM and for ANO from the wild type value of 0.02 to 0.8 mM, despite the opposing effects on $V_{max}$.

**Discussion**

Several spectroscopic studies on R. sphaeroides DMSO reductase have resulted in significant advances in the understanding of the structure and chemistry of molybdenum enzymes. The recent discovery of the bis(MGD) form of the molybdenum cofactor (8) and the elucidation of the x-ray crystal structure of DMSO reductase (9) along with the information derived from other spectroscopic studies including EXAFS (6), EPR (3, 5), RR (7), and MCD (5) have laid the foundation for further advances in the understanding of the structure and function of more complex molybdoenzymes.

The formation of mature DMSO reductase involves two major processes: attachment of the complex bis(MGD)molybdenum cofactor to the protein and transport of the protein to the periplasm with removal of the N-terminal signal peptide. Knäblein et al. (20) resorted to homologous expression of R. capsulatus DMSO reductase in Rhodobacter on the basis that E. coli is unable to supply the complex bis(MGD)molybdenum cofactor. The data presented here show that their conclusion is incorrect and that Rhodobacter DMSO reductase can in fact be expressed in E. coli in sufficient amounts for extensive structural and spectroscopic characterization. We also find that the coding sequence of the mature protein is sufficient to produce a fully functional enzyme, demonstrating that attachment of the cofactor does not require translocation of the protein into the periplasm. In fact, these data suggest the holoprotein is formed prior to translocation into the periplasmic compartment, a result consistent with recent work by Santini et al. (39) demonstrating that acquisition of the molybdenum cofactor in the cytoplasm of E. coli is a prerequisite for translocation of TMAO reductase into the periplasm.

E. coli cells contain both MPT and MGD. Earlier studies from our laboratory showed rat liver sulfite oxidase heterologously expressed in E. coli contained only MPT, its normal pterin, and no MGD (23). In the present studies we find that heterologously expressed DMSO reductase contains only MGD and no MPT. In combination, these observations show that even in the case of exogenous molybdoenzymes there is rigorous specificity with respect to the form of pterin incorporated. The stoichiometry of molybdenum and MGD in the recombinant R. sphaeroides DMSO reductase also compares very favorably with that of enzyme purified from R. sphaeroides cells. In contrast, purified recombinant E. coli DMSO reductase homologously expressed in E. coli was found to contain only about 28% of the expected amount of molybdenum cofactor (40). The much higher cofactor occupancy in our preparations is a definite advantage, and indeed a necessity, for high quality crystallographic data.

One surprising result of heterologous expression of DMSO reductase in E. coli was the obvious difference in color between the recombinant enzyme as purified and protein isolated from R. sphaeroides. Since a single turnover converts the isolated, recombinant enzyme to the native state and form, the difference between the two forms is not distinguishable by steady state kinetic analysis. However, detailed EXAFS studies indicate that the isolated green recombinant enzyme initially contains a dioxo-molybdenum structure with no Mo–O bond, suggesting that its conversion to the brown protein with the native absorption spectrum involves loss of one of the oxo groups concomitant with the mooring of the molybdenum to Ser-147 ligand (41). This finding demonstrates a plasticity of the molybdenum-active site that may explain the structural variations previously observed. Other heterologously expressed molybdenum enzymes may also require a reduction-oxidation cycle to convert an anomalous molybdenum coordination field to the native form.

In addition to the ability to produce recombinant R. sphaeroides DMSO reductase in E. coli cells, the work presented here involving the S147C variant demonstrates the anticipated

suitability of DMSO reductase for the creation of selected mutations in the coding sequence of the enzyme followed by efficient expression in E. coli cells and rapid, activity-independent purification to homogeneity. The residue known or expected to provide a ligand to the molybdenum has been subjected to site-directed mutagenesis in other enzymes, providing a variety of results. An analogous Ser to Cys variant was recently created in the catalytic subunit of the E. coli DMSO reductase (42), but the purified enzyme was essentially inactive. The effect of the mutation on the absorption spectrum of the molybdenum center could not be assessed, since the protein also contains Fe/S centers. In addition, EPR studies on the mutant demonstrated a heterogeneous ligand field. Molybdenum ligands have also been altered in enzymes that do not contain the bis(MGD) form of the molybdenum cofactor with resultant loss of activity. The Cys-207 molybdenum ligand of human sulfite oxidase has been replaced with Ser, and although the resulting variant contained a novel trioxo Mo(VI) center, it possessed no activity (43, 44). The replacement of Cys-150 of Aspergillus nidulans nitrate reductase, expected to be a ligand to the molybdenum, with an Ala residue, yielded an inactive protein (45). On the other hand, the replacement of the selenocysteine ligand of E. coli formate dehydrogenase H with a Cys produced a variant that retained formate dehydrogenase activity, although the $k_{cat}$ was significantly decreased (10, 46). The purified variant described in this work retains about 40% of the DMSO reductase activity of the wild type enzyme and has reproducible oxidized and reduced absorption spectra that differ from those of the native enzyme. Additionally, EXAFS (41) and x-ray crystallography have shown that the side chain sulfur of the Cys-147 variant does in fact become a molybdenum ligand.

An interesting result of the S147C substitution is the variable effect on the activity of the enzyme toward alternative substrates. The substitution leads to losses of 61 and 79% of DMSO and TMAO reductase activities, respectively, and a nearly complete loss of ability to reduce MetSO, chlorate, and BSO. However, it increases the ANO-reducing activity of the enzyme by greater than 400%. In fact, the ANO reductase activity of the S147C variant exceeds the activity of the wild type enzyme toward any other substrate, exceeding the TMAO reductase activity 2-fold and the DMSO reductase activity 10-

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**TABLE IV**

Absorption maxima of recombinant, wild-type, and S147C DMSO reductase

<table>
<thead>
<tr>
<th>Sample (^a)</th>
<th>Color</th>
<th>$\lambda_{\text{nm}}$</th>
<th>$\epsilon$</th>
<th>$\lambda_{\text{nm}}$</th>
<th>$\epsilon$</th>
<th>$\lambda_{\text{nm}}$</th>
<th>$\epsilon$</th>
<th>$\lambda_{\text{nm}}$</th>
<th>$\epsilon$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rOX</td>
<td>Green</td>
<td>380</td>
<td>3600</td>
<td>470</td>
<td>2400</td>
<td>650</td>
<td>1700</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rREOX</td>
<td>Brown</td>
<td>355</td>
<td>5300</td>
<td>470</td>
<td>2800</td>
<td>550</td>
<td>2000</td>
<td>720</td>
<td>2500</td>
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<tr>
<td>wtMo(VI)</td>
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<td>355</td>
<td>6000</td>
<td>470</td>
<td>2900</td>
<td>550</td>
<td>2200</td>
<td>720</td>
<td>2500</td>
</tr>
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<td>345</td>
<td>8200</td>
<td>405</td>
<td>4500</td>
<td>515</td>
<td>2700</td>
<td>695</td>
<td>1500</td>
</tr>
<tr>
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<td>Pink</td>
<td>345</td>
<td>8200</td>
<td>405</td>
<td>4500</td>
<td>515</td>
<td>2700</td>
<td>695</td>
<td>1500</td>
</tr>
</tbody>
</table>

\(^a\) DMSO reductase samples: purified recombinant (rOX), redox-cycled recombinant (rREOX), Mo(VI) DMSO reductase from R. sphaeroides cells (wtMo(VI)), S147C oxidized (mOX), and S147C reduced (mRED).

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**FIG. 4.** Absorption spectra of recombinant DMSO reductase. Top, 5 mg/ml recombinant R. sphaeroides DMSO reductase from BL21(DE3) cells as purified (---) and after reduction with reduced methyl viologen and reoxidation with Me$_2$SO (---). Bottom, 5 mg/ml methyl viologen-reduced, Me$_2$SO-reoxidized recombinant R. sphaeroides DMSO reductase from BL21(DE3) cells (---) and 5 mg/ml DMSO reductase purified from R. sphaeroides cells (---).

**FIG. 5.** Absorption spectra of the S147C R. sphaeroides DMSO reductase variant. Top, 5 mg/ml oxidized (---) and methyl viologen-reduced (---) S147C R. sphaeroides DMSO reductase from BL21(DE3) cells. Middle, 5 mg/ml oxidized S147C DMSO reductase and as-purified recombinant DMSO reductase from BL21(DE3) cells. Bottom, 5 mg/ml oxidized S147C DMSO reductase and methyl viologen-reduced, Me$_2$SO-oxidized recombinant DMSO reductase from BL21(DE3) cells.

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\(^4\) Hermann Schindelin, personal communication.
Re-design of *R. sphaeroides* Reductase

### Table V

| Substrate | Mo per molecule | Guanine per molecule | Specific activity
|-----------|-----------------|----------------------|-----------------
| rDR1      | 0.76            | 85                   | 0.98            | 2.4          | 2.6          | 80          | 3.4          | 93            |
| rDR2      | 0.71            | 79                   | 1.7             | 89           | 2.4          | 2.6          | 80           | 3.7          | 100           |
| mDR1      | 0.87            | 97                   | 1.7             | 91           | 2.0          | 1.2          | 36           | 1.4          | 37            |
| mDR2      | 0.66            | 74                   | 1.9             | 100          | 2.9          | 0.98         | 30           | 1.5          | 41            |
| wtDR      | 0.90            | 100                  | 1.9             | 100          | 2.1          | 3.3          | 100          | 3.7          | 100           |

a Atoms of molybdenum per molecule of enzyme.

b Percent relative to wild type.

c Molecules of guanine per atom of molybdenum.

d Units of DMSO reductase activity per nmol of enzyme.

### Table VI

| Substrate | Activity<br>units/nmol Mo | Change<br>%<br>Wild type | Relative activity<br>Wild type | Relative activity<br>S147C
|-----------|---------------------------|--------------------------|------------------|------------------
| Me2SO     | 3.6                       | -61                      | 100              | 39               |
| TMAO      | 18                        | -79                      | 500              | 107              |
| MetSO     | 4.3                       | -100                     | 120              | 0.11             |
| Chlorate  | 5.5                       | -97                      | 150              | 5.3              |
| BSO       | 4.8                       | -99                      | 130              | 0.72             |
| ANO       | 7.1                       | 36                       | 407              | 200              |

Effect of the S147C substitution on specific activity with various substrates

| Substrate | Activity<br>units/nmol Mo | Change<br>%<br>Wild type | Relative activity<br>Wild type | Relative activity<br>S147C
|-----------|---------------------------|--------------------------|------------------|------------------
| Me2SO     | 3.6                       | -61                      | 100              | 39               |
| TMAO      | 18                        | -79                      | 500              | 107              |
| MetSO     | 4.3                       | -100                     | 120              | 0.11             |
| Chlorate  | 5.5                       | -97                      | 150              | 5.3              |
| BSO       | 4.8                       | -99                      | 130              | 0.72             |
| ANO       | 7.1                       | 36                       | 407              | 200              |

### References

