Rhodobacter sphaeroides f. sp. denitrificans biotin sulfoxide reductase has been heterologously expressed in *Escherichia coli* as a functional 106-kDa glutathione S-transferase fusion protein. Following cleavage with Factor Xa and purification to homogeneity, the soluble 83-kDa enzyme retained biotin sulfoxide reductase activity using reduced methyl viologen or reduced benzyl viologen as artificial electron donors. Initial rate kinetics indicated a specific activity at pH 8.0 of 0.9 μmol of biotin sulfoxide reduced per min/nmol of enzyme and *Km* values of 29 and 15 μM for reduced methyl viologen and biotin sulfoxide reductase, respectively. Biotin sulfoxide reductase was also capable of reducing nicotinamide-N-oxide, methionine sulfoxide, trimethylamine-N-oxide, and dimethyl sulfoxide, although with varying efficiencies, and could directly utilize NADPH as a reducing agent, both for the reduction of biotin sulfoxide and ferricyanide. The enzyme contained the prosthetic group, molybdopterin guanine dinucleotide, and did not require any accessory proteins for functionality. These results represent the first successful heterologous expression and characterization of a functional molybdopterin guanine dinucleotide-containing enzyme and the demonstration of reduced pyridine nucleotide-dependent biotin sulfoxide reductase activity.

Biotin sulfoxide (BSO) reductase, which catalyzes the reduction of d-biotin d-sulfoxide to d-biotin according to the following scheme,

\[
d\text{-Biotin \ s-sulfoxide} + 2e^- + 2H^+ \rightarrow d\text{-biotin} + H_2O \quad (\text{Eq. 1})
\]

has been postulated to function in a variety of roles in bacterial metabolism (Pierson and Campbell, 1990) including scavenging biotin sulfoxide from the environment and thus allowing bacteria to utilize this oxidized form of biotin for biosynthetic reactions: reducing bound intracellular biotin, such as bound to biotin-containing carboxylases or protein degradation products, that have become oxidized in aerobic environments; or as a potential protector of the cell from oxidative damage similar to the proposed roles of methionine sulfoxide reductase (Ejiri et al., 1980; Brot et al., 1981; Rahman et al., 1992) and superoxide dismutase (Fridovich, 1989).

BSO reductase has been partially purified from *Escherichia coli* and demonstrated to be a soluble protein that requires an unidentified form of the Mo cofactor (Rajagopalan and Johnson, 1992) and several accessory proteins for activity (del Campo-Campbell and Campbell, 1982). These accessory proteins include a small, heat stable, thioredoxin-like protein moiety, referred to as protein-(SH)2, and which functions as a source of reducing equivalents and an unidentified flavoprotein (del Campo-Campbell et al., 1979). The extensive characterization of BSO reductase has been limited by the low natural abundance of the protein, its constitutive expression, and the requirement for auxiliary proteins for activity coupled with the difficulty of its detection in the absence of specific antibodies. While the *E. coli* BSO reductase bistC structural gene has been cloned and sequenced, the enzyme has not been produced using heterologous expression systems.

Biotin sulfoxide reductase, either from *E. coli* or *Rhodobacter sphaeroides* (Pollock and Barber, 1995), together with *R. sphaeroides* Me₂SO reductase (Barber et al., 1995) and *E. coli* trimethylamine N-oxide reductase (Mejean et al., 1994) are unique members of the small class of metalloenzymes that require Mo for functionality as their sole prosthetic group. Previous attempts to develop *E. coli*-based heterologous expression systems for this group of MGD-containing enzymes has been limited to *R. sphaeroides* Me₂SO reductase, where the enzyme was produced in an insoluble and inactive form (Hilton and Rajagopalan, 1996a).

We have heterologously expressed the *R. sphaeroides* BSO reductase as a GST fusion protein (Smith and Johnson, 1988) facilitating its isolation as a homogeneous, functional enzyme that contains MGD as its sole prosthetic group. Initial rate kinetic studies have demonstrated that the enzyme can use a variety of substrates, in addition to BSO and have established for the first time that the enzyme can also directly use NADPH as an efficient electron donor.

**MATERIALS AND METHODS**

**Chemicals, Enzymes, and Reagents**

Restriction enzymes and Factor Xa protease were purchased from Promega (Madison, WI), U. S. Biochemical Corp., and New England Biolabs (Beverly, MA). Media for bacterial growth was purchased from Difco, and aprotinin, nucleotide pyrophosphatase (type III), antibiotics, biotin, thiamine hydrochloride, methyl viologen, benzyl viologen, p-dimethylaminocinnamaldehyde, reduced glutathione, reduced thioredoxin (*Spirulina* sp.) and basic buffer chemicals were purchased from...
Sigma. Agarose, uren, SDS, acrylamide and bisacrylamide, protein molecular weight markers, as well as protein assay solution, were purchased from Bio-Rad. Isopropyl-β-D-galactoside was purchased from Research Products International Corp. (Mt. Prospect, IL) and ProBlott™ nylon membrane was purchased from Applied Biosystems.

**Vectors**

pUC19 was purchased from Boehringer Mannheim and the GST fusion protein expression vector, pGEX-5X-2, was purchased from Pharmacia Biotech Inc.

**GST-BSO Reductase Expression Vector Construction**

The entire 2.4-kilobase BSO reductase coding region was excised from the pUC19 construct (Pollock and Barber, 1995) using EcoRI and SaII restriction enzymes and cloned into the EcoRI and SaII sites of the pGEX-5X-2 expression vector. DNA sequencing was performed on this construct to confirm the proper junction sites and reading frame for the pGEX-5X-2 expression vector. DNA sequencing was performed on this construct to confirm the proper junction sites and reading frame for the BSO reductase gene using the Sequenase Version 2 (U. S. Biochemical Corp.) sequencing kit and [α-35S]dATP.

**Protein Expression and Purification**

Overnight cultures of E. coli JM109 transformed with either the pGEX-5X-2 vector containing the BSO reductase sequence, or the vector alone as a control, were diluted 1/100 in LB supplemented with ampicillin (100 μg/ml final concentration) and sodium molybdate (1 mM final concentration) and grown at 37 °C to an OD600 of 0.8. Isopropyl-β-D-galactoside (0.1 mM final concentration) was added to induce protein expression and the cells were grown for an additional 4 h at 24 °C. The cells were harvested by centrifugation, resuspended in phosphate-buffered saline supplemented with dithiothreitol (10 mM), sodium molybdate (1 mM), aprotinin (0.1 mg/ml), EDTA (1 mM), and phenylmethylsulfonyl fluoride (0.1 mM), and sonicated on ice. The sonicated cells were centrifuged at 30,000 × g for 30 min and the supernatant chromatographed on a glutathione-agarose affinity column (8-ml matrix volume) pre-equilibrated with phosphate-buffered saline. Following several washes with phosphate-buffered saline, the bound fusion protein was eluted with 50 mM Tris-HCl buffer, containing 10 mM GSH, pH 8. Fractions exhibiting MV:BSOR activity were concentrated by pressure filtration (PM-10, Amicon) and stored in liquid nitrogen. Following cleavage with Factor Xa, the affinity-purified BSO reductase complex was re-chromatographed using glutathione-agarose to remove the GST portion of the fusion protein. Further purification of the BSO reductase to separate the enzyme from auxiliary proteins was achieved utilizing fast protein liquid chromatography gel filtration on a Superose 12 column (1 × 30 cm, Pharmacia) in 50 mM Tris-HCl buffer, pH 8, and anion exchange on a Mono-Q column (0.5 × 5 cm) utilizing a salt gradient from 0 to 0.5 M NaCl.

Untransformed E. coli JM 109 and ΔMumu29 cells were grown in LB to an OD600 of 2.0, respectively. Cells were harvested and disrupted using identical methods to those described for the transformed cells and the supernatants assayed for both NADPH:BSOR and BSO-independent NADPH-oxidation activities, prior to and following dialysis, in 50 mM Tris, pH 8. R. sphaeroides cells were grown either aerobically in LB or anaerobically in the presence of 0.2% nitrate to an OD600 of 2.5, harvested, and processed as described for the E. coli cells. R. sphaeroides Me3SO reductase was isolated as described previously (Barber et al., 1995).

**Factor Xa Proteolysis**

Protein samples were subjected to cleavage by Factor Xa (1% w/v) in 50 mM Tris-HCl buffer, containing 150 mM NaCl and 1 mM CaCl2, pH 8, at 16°C for 16 h.

**Protein Analysis**

BSO reductase purity was assessed by both SDS-PAGE and reverse-phase HPLC analysis. Enzyme samples (1–10 μg of total protein) were analyzed using either 10 or 12.5% SDS-PAGE gels (Laemmli, 1970) stained with Coomassie Blue. For reverse-phase HPLC analysis, BSO reductase (30 μg) was mixed with an equal volume of 8 μg guanidine hydrochloride, acidified with 0.5% acetic acid, and chromatographed on a C4 column (4.6 × 30 mm) using a linear gradient of trifluoroacetic acid (0.1%) to trifluoroacetic acid (0.1%), acetonitrile (70%).

**Molybdenum Cofactor Analysis**

Samples of BSO reductase and Me3SO reductase were denatured with 1% SDS for 12 h followed by boiling for 20 min. The SDS was removed by precipitation with KCl (0.250 M), the samples centrifuged at 20,000 × g for 5 min, and the denatured protein separated from the cofactor utilizing ultrafiltration spin columns (ultrafree-MC® 5,000 MW cut-off, Millipore Corp.). The cofactor-containing solution was assayed for protein and its UV visible and fluorescence spectra recorded. For nucleotide pyrophosphatase cleavage, MgCl2, and lyophilized pyrophosphate were added to the cofactor-containing sample. The sample was incubated at 37 °C for 15 min following which the cofactor was separated from the pyrophosphatase by ultrafiltration.

**Protein Sequencing**

NH2-terminal sequencing was performed as described by Pollock and Barber (1995). For the generation and sequencing of internal peptides, protein samples were separated by SDS-PAGE, translated onto nylon membranes and subjected to in situ proteolytic cleavage. Excised membrane sections were first blocked with polyvinyl pyrrolidone (1% in water) for 30 min, extensively washed with water and immersed in 100 mM of protease digestion buffer (0.1 M-Tris-HCl, pH 8, 1% Triton X-100 and 10% acetonitrile). Endoprotease Lys-C (1 μg) was added, and digestion continued at 37 °C overnight. Following proteolysis, the peptide containing solution was denatured with guanidine hydrochloride, acidified with acetic acid, and individual peptides separated by reverse-phase HPLC (Vydac C18 column, 4.6 × 250 mm) as described by Neame and Barber (1989).

**Spectroscopic Analysis**

UV-visible spectra were obtained using a Shimadzu Scientific Inst. Inc. (Columbia MD) UV2101PC spectrophotometer and fluorescence spectra were obtained using a JASCO (Easton, MD) FP770 spectrofluorimeter. UV CD spectra were obtained using a JASCO J7T10 spectrocolorimeter as described by Trimble et al. (1996).

**Enzyme Activities**

**Biotin Analysis Using Reverse-phase HPLC—** The conversion of BSO to biotin was performed using MES buffer, pH 6 (140 μl of 114 mM), BSO (50 μl of 5 mg/ml), MV (500 μl of 100 mM in 50 mM Tris buffer, pH 7.5), and prepared with H2 and Pt-asbestos) and homogeneous BSO reductase (30 μl of 0.5 mg/ml). The buffer solution containing BSO was made anaerobic and extensively buffered with argon. MV2 was added following the BSO reductase and the reaction allowed to proceed anaerobically until the MV2 had been depleted (about 5–10 min, final solution pH of 8). The reaction mixture was filtered (5,000 M2 cut-off ultrafiltration spin column) to separate the protein and reaction products and the latter subsequently analyzed by reverse-phase HPLC (Vydac C18 column, 4.6 × 250 mm) using a linear gradient of trifluoroacetic acid (0.05%, pH 2.5) to trifluoroacetic acid (0.05%, pH 2.5), acetonitrile (70:30, v/v) at a flow rate of 1 ml/min. Control reactions contained MV2 and buffer; BSO, MV2 and buffer; biotin (50 μl of 5 mg/ml), MV2, and buffer; BSO, MV2, heat-inactivated BSO reductase, and buffer, respectively.

**Disk Microbiological Assay—** The disk microbiological assay was performed using a modified version of that previously described by Pollock and Barber (1995). E. coli ΔMumu29 (bio-, bis-, thi-), which cannot grow in the absence of biotin or in the presence of BSO and absence of biotin, was deposited onto minimal glucose plates supplemented with tetrazolium (0.0116%, thi (10 μg/ml), and str (75 μg/ml). The conversion of BSO to biotin was performed as described for the reverse-phase HPLC analysis of the BSO reductase reaction and the reaction products separated from the protein by ultrafiltration. Following dilution with water, aliquots (25 μl) of each reaction product were applied to filter disks placed on top of the minimal glucose plates containing E. coli ΔMumu29. The plates were incubated at 37°C overnight and inspected for bacterial growth, detectable as the formation of red colonies surrounding the filter circles.

**Spectrophotometric Assays—** BSO reductase activities were routinely determined at 600 nm under anaerobic conditions at 25 °C in 50 mM Tris buffer, pH 8, as the oxidation of either MV2 (115 μM) or BV2 (115 μM) in the presence of BSO (1.7 mM). Concentrations of MV2 or BV2 were calculated using extinction coefficients of 13 mm-1 cm-1 (ε600 nm) (Thoruneley, 1974) and 13 mm-1 cm-1 (ε550 nm) (Liassolo et al., 1984), respectively.
**RESULTS**

*Expression of the GST-BSO Reductase Fusion Protein*

The results of expression studies using *E. coli* harboring the GST-BSO reductase expression vector are shown in Fig. 1. Optimal expression of the soluble form of the GST-BSO reductase fusion protein was obtained using low concentrations of isopropylthio-β-galactoside (0.1 mM) coupled with bacterial culture at room temperature for 5 h. Elevated temperatures or longer growth periods resulted in decreased production of the soluble fusion protein and a corresponding increase in the formation of inclusion bodies. Under optimum expression conditions and compared to control cells containing the GST expression vector without the BSO reductase gene, the GST-BSO reductase fusion protein could be detected as the presence of an additional protein band of molecular mass approximately 106 kDa in both the pellet (Fig. 1, *lane P*) and soluble (Fig. 1, *lane S*) protein fractions. Quantitation of protein expression levels suggested that the soluble GST-BSO reductase fusion protein represented 2–3% of the total soluble protein while the insoluble form represented 8–10% of the pellet fraction. In comparison, control cells containing the vector alone produced a major, soluble protein with a molecular mass of 26 kDa, that exhibited GST activity (data not shown).

**BSO Reductase Isolation**

Under optimal conditions, the yield of purified BSO reductase protein corresponded to 1 mg from 8 liters of cells (8 g). The individual steps utilized to purify the BSO reductase to homogeneity together with the respective yields are indicated in Table I. The initial affinity purification of the soluble *E. coli* cell extract using glutathione-agarose resulted in the isolation of a complex protein mixture that exhibited both GST activity and MV:BSOR activity indicating the presence of the required fusion protein. Examination of the protein composition of this sample indicated the presence of four proteins, detected as individual bands corresponding to molecular masses of 106, 70, 60, and 26 kDa, respectively, following SDS-PAGE analysis (Fig. 1, *lane A, u*). The high molecular mass band, corresponding to a molecular mass of 106 kDa, that occasionally appeared as a doublet following SDS-PAGE, was subsequently identified as the GST-BSO reductase fusion protein by a combination of Factor Xa proteolysis and its retention of both GST and MV:BSOR activity. However, this band was usually partially proteolysed even without the addition of Factor Xa, evident as the presence of the small 26-kDa GST fragment. Following proteolysis with Factor Xa (Fig. 1, *lane A, c*), the 106-kDa band was converted to two bands with apparent molecular masses of 80 and 26 kDa, respectively, the former retaining MV:BSOR activity and the latter retaining GST activity. Direct analysis of the amino terminus of the 80-kDa protein yielded the sequence shown in Table II, confirming the identity of this protein as BSO reductase while the 26-kDa protein corresponded to the cleaved GST expression tag.

Analysis of the affinity-purified GST-BSO protein complex indicated that two additional protein components, corresponding to molecular masses of 70 and 60 kDa, respectively, consistently co-purified with the fusion protein. These two additional proteins were tightly associated since they were only partially resolved by fast protein liquid chromatography gel filtration of the complex (Fig. 1, *lanes B and C*).

To confirm that the two auxiliary proteins bound specifically to BSO reductase and not the intact fusion protein, the isolated GST-BSO reductase fusion protein complex (Fig. 2, *lane A*) was treated with Factor Xa and affinity purified using glutathione-agarose. The flow-through from the column contained BSO reductase, identified by the retention of MV:BSOR activity, and both the 70- and 60-kDa auxiliary proteins (Fig. 2, *lane B*), whereas the cleaved GST portion remained bound to the column and could subsequently be eluted with GSSH as the sole protein (Fig. 2, *lane C*). BSO reductase could be resolved from the auxiliary proteins by ion exchange chromatography yielding a homogeneous enzyme (Fig. 2, *lane D*).

The purity of the isolated BSO reductase obtained following ion exchange and gel filtration chromatography was also examined using reverse-phase HPLC. The HPLC chromatogram indicated the presence of a single protein peak corresponding to an elution time of 40.5 min (data not shown).

**Auxiliary Protein Identification**

To identify the 60-kDa auxiliary protein that bound to BSO reductase, the amino terminus of the protein was examined yielding the sequence shown in Table II. This sequence was identical to the first 15 residues (AAKDVKFHPNDARKVM) of the *E. coli* groEL protein (Hemmesgen et al., 1988). In contrast, we were unable to obtain any reliable sequence from the amino-terminal region of the 70-kDa protein. However, following proteolysis, three internal peptides were obtained which yielded the sequences shown in Table II, confirming the identity of this protein as *E. coli* hsp70 (Bardwell and Craig, 1984) and corresponding to residues 93–106 (IIAADNGDAWVEVK) and 587–594 (KMQELAQV) and 598–616 (LMEIAQQHQAQQQTAGADA), respectively. Attempts to increase the level of soluble BSO reductase expression, by co-transformation of the
Biotin Sulfoxide Reductase

**Purification of recombinant R. sphaeroides BSO reductase**

| Purification step            | Activity | Specific activity | Recovery | Fold |
|-----------------------------|----------|-------------------|----------|------|
|                             | NADPH | MV⁺ | Protein | NADPH | MV⁺ | %       |         |
| Cell supernatant            | 520    | 15.6 | 81.0    | 6.42  | 0.193 | 100     | 1.00    |
| Affinity chromatography     | 400    | 12.0 | 4.60    | 87.0  | 2.61  | 77      | 13.5    |
| Factor Xa cleavage          | 348    | 10.4 | 3.10    | 112   | 3.35  | 67      | 17.4    |
| Anion exchange              | 331    | 9.93 | 1.05    | 315   | 9.46  | 64      | 49.0    |
| Gel filtration              | 322    | 9.66 | 1.00    | 322   | 9.66  | 62      | 50.1    |

* Units: μmol of NADPH consumed/min (NADPH:BSO reductase activity).
  b Units: μmol of BSO consumed/min (MV:BSO reductase activity).

**Amino acid sequences obtained for BSO reductase and auxiliary proteins**

| Protein          | Molecular mass (kDa) | Sequence identity | Sequence |
|------------------|----------------------|-------------------|----------|
| BSO reductase    | 80                   | Amino terminus    | YPITRVPH |
| hsp70            | 70                   | Internal peptides | HIAADNGDAWVEYK |
| groEL            | 60                   | Amino terminus    | LMEIAQQQHAQQQQTAGADA |

**UV-visible spectral characteristics of BSO and Me₂SO reductases**

| Protein          | λ₃75 | λ₃20 | λ₅20 | λ₅75 |
|------------------|------|------|------|------|
| BSO reductase    | 0.590| 0.033| 0.673| 0.082|
| Me₂SO reductase  | 0.591| 0.038| 0.686| 0.089|

**Cofactor Analysis**

To identify the Mo cofactor bound to the recombinant BSO reductase, the purified enzyme (Fig. 2, lane D) was denatured with SDS and the cofactor isolated. The UV-visible absorbance spectrum of the isolated Mo cofactor is shown in Fig. 3, together with the spectrum obtained from a corresponding sample of R. sphaeroides Me₂SO reductase, a known MGD-containing enzyme (Johnson et al., 1990). The spectrum of the isolated cofactor exhibited an additional peak at 249 nm due to absorption by the 5'-GMP portion of the cofactor. Identical results have previously been reported for Me₂SO reductase by Johnson et al. (1990).

To confirm the identity of the Mo cofactor as MGD, fluorescence emission spectra of the isolated cofactor, both prior to and following pyrophosphatase cleavage, were obtained and compared to those from a sample of cofactor isolated from Me₂SO reductase. Incubation of the liberated MGD cofactor with pyrophosphatase, resulting in hydrolysis of the pyrophosphate linkage between the molybdopterin and 5'-GMP moieties, produced an increase in the fluorescence intensity (Table IV) of both samples with no shift in the fluorescence emission maxima which were determined to be 469 nm for the cofactor isolated from BSO reductase and 468 nm for MGD isolated from Me₂SO reductase.

**Enzyme Activities**

**HPLC Analysis**—The ability of the isolated BSO reductase to convert BSO to biotin, utilizing MV⁺ as an electron donor, was initially examined using reverse-phase HPLC analysis of the reaction products, as shown in Fig. 4. Under the conditions used for analysis, standard samples of BSO and biotin eluted at 9.2 min (peak 1, Trace C) and 18.7 min (peak 2, Trace B), respectively, while the large early peak present in all the elution profiles was due to the presence of MV. In the absence of BSO reductase or in the presence of heat-inactivated enzyme,
none of the substrate was reduced to biotin (Traces C and D, respectively). However, in the presence of BSO reductase (Trace E), approximately 50% of the BSO was converted to biotin as evident by the decrease in the amplitude of the peak at 9.2 min and the appearance of the peak at 18.7 min due to biotin formation.

HPLC analysis was also utilized to examine the conversion of BSO to biotin catalyzed by BSO reductase using reduced glutathione and reduced thioredoxin as potential electron donors. Incubation of BSO reductase (5 μg) and BSO (1.6 mM) for 2 h at 25 °C in 50 mM Tris buffer, pH 8, in the presence of reduced glutathione (10 mM) or reduced thioredoxin (1 mM) and EDTA (1 mM), respectively, followed by HPLC analysis of the reaction mixture failed to indicate the formation of biotin, suggesting both reducing agents failed to function as suitable electron donors for BSO reductase.

**Disk Microbiological Assay**

To confirm that the BSO reductase reaction product eluting at 18.7 min on reverse-phase HPLC was biotin, we utilized this reaction product as well as control samples of BSO and biotin, in the disk biological assay as shown in Fig. 5. *E. coli* ΔMu29 mutants were unable to grow utilizing either the products of the reaction between MV⁺ and BSO (Disk c), the products of the reaction between MV⁺, BSO, and heat-inactivated enzyme (Disk a), MV⁺ (Disk d) or BSO in water (Disk f). In contrast, the ΔMu29 mutants grew, visible as red halos surrounding the disc, in either the presence of the products of the reaction between MV⁺, BSO, and BSO reductase (Disk b), MV⁺ and biotin (Disk e), or biotin in water (Disk g). Thus the test organism grew well only around the two disks containing authentic biotin or the product of the BSO reductase-catalyzed reaction.

**Initial Rate Kinetics**—The results of the previous two assays indicated that MV⁺ could function as a facile electron donor for BSO reductase and therefore could also be used in the first direct spectrophotometric assay for BSOR activity. Analysis of the initial rate measurements of the consumption of MV⁺ in the presence of BSO and several alternative electron acceptors yielded the kinetic constants shown in Table V.

Under the conditions of the assay, all Lineweaver-Burk plots were linear over the entire range of donor and acceptor substrate concentrations examined except for assays performed in the presence of high concentrations of BSO (>2 mM), nicotinamide N-oxide (>5 mM), and methionine sulfoxide (>2.5 mM)
where pronounced substrate inhibition was observed. At pH 8.0 and using 50 mM Tris buffer, BSO reductase yielded a $V_{\text{max}}$ of 0.9 μmol of BSO consumed/min/nmol enzyme and $K_m$ values of 29 and 15 μM for MV$^+$ and BSO, respectively. MV$^+$ could be replaced by BV$^+$ with no change in $V_{\text{max}}$ (0.9 μmol of BSO consumed/min/nmol of enzyme) but an increase in $K_m$, to 35 μM, suggesting the enzyme exhibited very little specificity with respect to the nature of the artificial electron donor. However, the enzyme could also utilize additional oxidizing substrates including nicotinamide-N-oxide, methionine sulfoxide, trimethylamine N-oxide, and Me$_2$SO with comparable rates to that obtained with BSO although the $K_m$ values for these substrates varied from a low of 20 μM (nicotinamide-N-oxide) to a high of 14.4 mM (Me$_2$SO). However, values for $V_{\text{max}}/K_m$ indicated BSO reductase exhibited a marked preference for BSO as the oxidizing substrate.

Equivalent kinetic constants were also obtained for the MV:BSOR activity of the isolated GST-BSO reductase fusion protein indicating that the GST domain had no influence on the catalytic activity of the BSO reductase domain.

**NAD(P)H-dependent BSO Reductase Activity**

The presence of a putative ATP/GTP-binding P-loop in the BSO reductase sequence (Pollock and Barber, 1995), prompted the examination of reduced pyridine nucleotides as potential electron donors for the enzyme. Under aerobic conditions and in the absence of BSO, consumption of either NADPH or NADH by BSO reductase was not observed. In contrast, in the presence of BSO, rapid consumption of NAD(P)H was detected resulting in the conversion of BSO to biotin which was subsequently confirmed by both HPLC analysis and the disk microbiological assay of the reaction mixture. BSO reduction was dependent on the presence of the enzyme since in its absence or in the presence of heat-inactivated enzyme, no NADPH was consumed or biotin produced. The results of initial rate studies at pH 8.0, shown in Table VI, indicated that the purified enzyme exhibited a NADPH:BSO specific activity of 30 μmol of NADPH consumed/min/nmol of enzyme with $K_m$ values of 269 and 524 μM for NADPH and BSO, respectively. BSO reductase was also capable of utilizing NADH in place of electron donor, although with greatly reduced efficiency, the specific activity being reduced approximately 11-fold from that determined with NADPH while the $K_m$ for NADH was increased to 394 μM. NADPH also functioned as a suitable electron donor for the reduction of a variety of alternative oxidizing substrates (Table VI) although BSO remained the preferred substrate. Identical studies performed using purified *Rhodobacter sphaeroides* Me$_2$SO reductase revealed that in contrast to BSO reductase, Me$_2$SO reductase did not exhibit any reduced pyridine nucleotide dependent activity.

To determine if auxiliary proteins present in *E. coli* or *R. sphaeroides* cell lysates could enhance the activity of the purified BSO reductase, activities were examined in the presence and absence of either *E. coli* or *R. sphaeroides* cell extracts. Undialyzed cell lysates were observed to inhibit NADPH:BSOR activity approximately 2-fold, however, dialysis of the lysates to remove low molecular weight species resulted in no inhibition nor stimulation of enzyme activity, suggesting that in contrast to the *E. coli* BSO reductase the *R. sphaeroides* enzyme did not require any auxiliary proteins for functionality.

In the presence of NADPH, BSO reductase was also able to utilize ferricyanide as an artificial electron acceptor. At pH 8.0, the enzyme exhibited a NADPH:F$^+$ specific activity of 1.7 μmol of NADPH consumed/min/nmol of enzyme with $K_m$ values of 16 and 662 μM for NADPH and Fe(CN)$_6^{3-}$, respectively.

**DISCUSSION**

The preceding results demonstrate the first successful heterologous expression and isolation to homogeneity of a functional BSO reductase that contains MGD as its sole prosthetic group and which is catalytically active in the absence of any auxiliary protein components. In addition, we have provided the first demonstration that the enzyme can directly utilize reduced pyridine nucleotides as a source of reducing equivalents and also obtained initial rate kinetic constants that indicate that BSO reductase can utilize a variety of alternate oxidizing substrates in addition to *d*-biotin *d*-sulfoxide.

Expression of a recombinant BSO reductase as a GST fusion protein presented several potential advantages for the production of a functional molybdoprotein. First, the system allowed the affinity purification of the BSO reductase under reducing conditions which could be important when cells are broken by sonication to prevent damage of the Mo cofactor due to oxidation by free radicals, while second, glutathione-agarose has previously been used to isolate an active form of the Mo cofactor from xanthine oxidase under reducing conditions (Mendel and Alkulov, 1983) and therefore may stabilize the functional forms of Mo-containing enzymes during purification.

While the cloning of the BSO reductase has not resulted in very high levels of expressed soluble enzyme since the majority of the BSO reductase is produced as inclusion bodies, it has facilitated the isolation and purification of the enzyme to homogeneity in an active form in quantities sufficient for the first spectroscopic and kinetic characterization of the *Rhodobacter* enzyme.

### Table V

| Compound | $V_{\text{max}}$ (μmol/min/nmol enzyme) | $K_m$ (μM) | $V_{\text{max}}/K_m$ (μmol/min/nmol enzyme) |
|----------|--------------------------------------|------------|------------------------------------------|
| d-Biotin $d$-sulfoxide | 0.9 | 15 | $6.1 \times 10^{-2}$ |
| Nicotinamide N-oxide | 0.9 | 20 | $4.8 \times 10^{-3}$ |
| l-Methionine sulfoxide | 0.9 | 907 | $1.2 \times 10^{-3}$ |
| Me$_2$SO | 0.9 | 850 | $1.1 \times 10^{-3}$ |
| Trimethylamine N-oxide | 0.9 | 11,050 | $8.5 \times 10^{-5}$ |
| Dimethyl sulfoxide | 0.9 | 14,440 | $6.4 \times 10^{-5}$ |
| Reducing substrate | 0.9 | 29 | $3.1 \times 10^{-2}$ |
| MV$^+$ | 0.9 | 35 | $2.5 \times 10^{-2}$ |

### Table VI

| Compound | $V_{\text{max}}$ (μmol/min/nmol enzyme) | $K_m$ (μM) | $V_{\text{max}}/K_m$ (μmol/min/nmol enzyme) |
|----------|--------------------------------------|------------|------------------------------------------|
| d-Biotin $d$-sulfoxide | 30.0 | 524 | $5.7 \times 10^{-2}$ |
| Nicotinamide N-oxide | 19.5 | 414 | $4.8 \times 10^{-3}$ |
| l-Methionine sulfoxide | 6.2 | 10,000 | $6.0 \times 10^{-4}$ |
| Me$_2$SO | 6.1 | 10,070 | $6.0 \times 10^{-4}$ |
| Trimethylamine N-oxide | 30.8 | 482,000 | $1.0 \times 10^{-4}$ |
| Dimethyl sulfoxide | 9.6 | 207,000 | $4.6 \times 10^{-5}$ |
| Ferricyanide | 1.7 | 662 | $2.6 \times 10^{-3}$ |
| Reducing substrate | 30.0 | 269 | $1.1 \times 10^{-1}$ |
| NADH | 2.8 | 394 | $7.1 \times 10^{-3}$ |

$a$ 1 unit corresponds to 1 μmol of BSO consumed per min/nmol enzyme.

$b$ Oxidizing substrate specificity was determined using NADPH as the electron donor while reducing substrate specificity was determined using BSO as acceptor.
The isolation of a homogeneous BSO reductase has facilitated both cofactor analysis and limited structural comparisons. The enzyme has been demonstrated to contain MGD, which has also been shown to be the Mo cofactor present in R. sphaeroides Me₃SO reductase (Johnson et al., 1990) and a number of other related E. coli Mo-containing enzymes (Rajagopalan and Johnson, 1992). In addition, the spectroscopic and reverse-phase HPLC analyses of the purified enzyme have indicated the absence of any additional chromophores, confirming MGD to be the sole prosthetic group. Comparison of the UV CD spectra obtained for BSO reductase and Me₃SO reductase indicates the two proteins have similar conformations, which is also reflected in the extensive sequence conservation (Barber et al., 1995). These results are supported by the recently determined x-ray structure of R. sphaeroides Me₃SO reductase which has confirmed that this enzyme contains MGD as its sole prosthetic group and identified it as a mixed α/β protein in terms of its secondary structure (Schindelin et al., 1996).

Previous work on the E. coli BSO reductase resulted in the isolation of enzyme of undetermined purity (del Campillo-Campbell et al., 1979) while measurements of enzyme activity were limited to the use of the disk biological assay, performed in the presence of added cell lysate due to requirements for several auxiliary proteins. In contrast, the recombinant R. sphaeroides BSO reductase has been demonstrated to be fully functional in the absence of any additional auxiliary proteins. Comparison of the specific activity reported for the E. coli BSO reductase with that of the R. sphaeroides enzyme suggests that the latter is significantly more active (the specific activity reported for the E. coli enzyme corresponds to 1.6 × 10⁻⁵ μmol of BSO consumed/min/nmol of enzyme while the corresponding value for the R. sphaeroides enzyme is 9 × 10⁻¹ μmol of BSO consumed/min/nmol of enzyme). However, this substantial difference may be partly due to the greater purity of the latter enzyme and the use of an improved assay. While the enzyme was unable to utilize low molecular weight thiol compounds as electron donors, in agreement with previous genetic studies of E. coli BSO reductase (del Campillo-Campbell et al., 1979), reduced viologens were found to act as efficient reductants.

The isolation of a functional BSO reductase has been confirmed by three different assays: the direct analysis of the reaction products using reverse-phase HPLC, the disk microbiological assay, which indirectly confirms the presence of biotin as indicated by the growth of the test organism, and the development of a direct spectrophotometric assay for the enzyme-catalyzed oxidation of MV⁺ in the presence of BSO, similar to the method used to assay the activity of Me₃SO reductase (Bastian et al., 1991). The recombinant expression and isolation of a functional Mo-containing enzyme has previously only been described for rat hepatic sulfite oxidase (Garrett and Rajagopalan, 1994). The enzyme was expressed at low levels in E. coli, contained MPT, and retained sulfite:cytochrome c reductase activity, although no specific activities were reported. In contrast, recent attempts to express R. sphaeroides Me₃SO reductase in E. coli resulted in the production of the appropriately-sized insoluble recombinant protein which was devoid of Me₃SO reductase activity (Hilton and Rajagopalan, 1996a).

In contrast to either E. coli BSO reductase or R. sphaeroides Me₃SO reductase, we have demonstrated that R. sphaeroides BSO reductase can directly utilize reduced pyridine nucleotides as electron donors. Analysis of initial rate data has indicated a marked preference for NADPH when compared to NADH, the former yielding values of Vₘₐₓ/Kₘₐₓ directly comparable to those obtained using MV⁺ as the source of reducing equivalents. While both purified Me₃SO reductase and partially-purified E. coli BSO reductase are unable to directly use either NADPH or NADH as suitable reducing agents, the E. coli BSO reductase has been shown to exhibit limited NADPH-dependent BSOR activity in the presence of an auxiliary protein referred to as protein-S₂ reductase (del Campillo-Campbell et al., 1979).

This work has provided the first information concerning the oxidizing substrate specificity of BSO reductase. While the enzyme functions most efficiently with BSO, nicotinamide N-oxide, methionine sulfoxide, trimethylamine N-oxide, and Me₃SO could also be utilized as substrates although with varying efficiencies. While the enzyme was found to utilize nicotinamide N-oxide with a degree of efficiency comparable to that of BSO, markedly reduced efficiencies were observed for the remaining substrates. Although the turnover numbers for all five oxidizing substrates were comparable, the smaller substrates did not appear to bind as tightly to the enzyme as the larger or heterocyclic compounds. In addition, a similar substrate specificity was observed when using either MV⁺ or NADPH as the reducing agent. The MV⁺-dependent substrate specificity of BSO reductase appears to be the reverse of that for R. sphaeroides Me₃SO reductase where substrates such as trimethylamine N-oxide have been shown to bind less tightly than Me₃SO (Satoh and Kurihara, 1987). Comparison of the specific activities previously reported for R. sphaeroides Me₃SO reductase (Bastian et al., 1991; Hilton and Rajagopalan, 1996b) with that obtained for BSO reductase indicates that the two enzymes have comparable turnover numbers when utilizing Me₃SO or BSO as oxidizing substrates, respectively. In addition, our kinetic studies have indicated that BSO reductase can reduce methionine sulfoxide to methionine at a rate approximately 300-fold greater than that obtained for a similar reaction catalyzed by the E. coli enzyme, peptide methionine sulfoxide reductase (Rahman et al., 1992), that has been postulated to provide a repair mechanism for proteins that have been inactivated by oxidation. Thus, the ability of BSO reductase to reduce a variety of oxidized substrates may provide an additional pathway to reverse the results of oxidative damage.

Previous studies of protein expression in E. coli have indicated the involvement of chaperonins in promoting protein folding and inhibiting degradation of foreign proteins by ATP-dependent proteolysis. Thus the expression of a mutant form of alkaline phosphatase (pho61) in E. coli has demonstrated the association of the mutant protein with both hsp70 and grpE chaperonins (Sherman and Goldberg, 1992). For the expression of the fusion protein, CRAG, which contained sequences of cro, protein A, and a 14-amino acid portion of the lacZ gene product, the fusion protein was found to exist in two complexes with either hsp70 (Hellebust et al., 1989) or groEL (Sherman and Goldberg, 1991). groEL has also previously been shown to co-purify with a recombinant form of a plant glucosidase produced as a GST fusion protein (Keresztessey et al., 1996). In contrast, expression of BSO reductase has demonstrated the formation of a specific complex with both hsp70 and groEL and that the GST-BSO reductase fusion protein-chaperonin complex retained activity comparable to that of the purified enzyme suggesting retention of the native conformation in the fusion protein complex. While the precise role of these auxiliary chaperonin proteins in the expression of BSO reductase has not been identified, they may potentially function to assist in the correct folding or assembly of the recombinant protein.

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