Serine 121 Is an Essential Amino Acid for Biotin Sulfoxide Reductase Functionality*

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Biotin sulfoxide reductase (BSOR), EC 1.8.4.- catalyzes the reduction of d-biotin d-sulfoxide to d-biotin according to Scheme 1.

\[ \text{d-biotin d-sulfoxide} + 2e^- + 2H^+ \rightarrow \text{d-biotin} + H_2O \] (Scheme 1)

In prokaryotes, such as Escherichia coli (1) and the photosynthetic bacterium Rhodobacter sphaeroides f. sp. denitrificans biotin sulfoxide reductase (BSOR) catalyzes the reduction of d-biotin d-sulfoxide (BSO) to biotin, an important step in oxidized vitamin salvaging. In addition to BSO, the enzyme also catalyzes the reduction of a variety of other substrates, including methionine sulfoxide, with decreased efficiencies, suggesting a potential role as a general cell protector against oxidative damage. Recombinant BSOR, expressed as a glutathione S-transferase fusion protein, contains the molybdopterin guanine dinucleotide cofactor (MGD) as its sole prosthetic group, which is required for the reduction of BSO by either NADPH or reduced methyl viologen. Comparison of the amino acid sequences of BSOR and the closely related MGD-containing enzyme, dimethyl sulfoxide reductase, has indicated a number of conserved residues, including an active site serine residue, serine 121, which has been potentially identified as the fifth coordinating ligand of Mo in BSOR. Site-directed mutagenesis has been used to replace serine 121 with cysteine, threonine, or alanine residues in the BSOR sequence to assess the role of this residue in catalysis and/or Mo coordination. All three BSOR mutant proteins were expressed, purified to homogeneity, and demonstrated to contain both MGD by fluorescence spectroscopy and Mo by inductively coupled plasma mass spectrometry, similar to wild-type enzyme. However, all three mutant proteins were devoid of BSOR activity using either NADPH or reduced methyl viologen as the electron donor. These results strongly suggest that serine 121 in BSOR is essential for catalysis but is not essential for either Mo coordination or MGD binding.

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† The abbreviations used are: BSOR, d-biotin d-sulfoxide (BSO) reductase; GST, glutathione S-transferase; MGD, molybdopterin guanine dinucleotide; Me2SO, dimethyl sulfoxide; PCR, polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; FR, ferricyanide reductase; HPLC, high pressure liquid chromatography; MV2+, reduced methyl viologen radical cation; Mes, 4-morpholineethanesulfonic acid; amp, ampicillin.

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tase this residue corresponds to serine 147, whereas in BSOR the corresponding residue is serine 121. Mutation of Ser^{147} to a Cys in Me_{2}SO reductase has been shown to result in different- 
effect on substrate utilization. These include a loss of
approximately 61–100% of its activity toward substrates such as
Me_{2}SO and methionine sulfoxide and a >400% increase in
activity for adenosine 3'-oxide reduction (13).

To investigate the role of Ser^{121} in the functionality of BSOR,
we have generated the corresponding alanine, cysteine, and
threonine mutants and examined the proteins for both Mo and
MGD incorporation and their associated catalytic activities.

**Materials and Methods**

**Bacterial Strains**

*E. coli* JM 109 and DH5a were purchased from Promega (Madison, WI).

**Chemicals, Enzymes, and Reagents**

Factor Xa protease, *Pfu* DNA polymerase, and *Dpn*I restriction en-
zyme were purchased from Promega, and media for bacterial
growth was purchased from Difco. Restriction enzymes were purchased from
New England Biolabs Inc. (Beverly, MA). Aprotinin, antibiotics, biotin,
dithiothreitol, phenylmethylsulfonyl fluoride, reduced glutathione, and
basic buffer chemicals were purchased from Sigma, SDS, acrylamide,
bis-acrylamide, protein molecular weight markers, and protein assay
solution were purchased from Bio-Rad. Isopropylthio-
galactoside was obtained from Research Products International Corp. (Mt. Prospect,
IL). *d-Biotin* and *d*-sulfide was prepared as described by Pollock and
Barber (14).

**Site-directed Mutagenesis**

Site-directed mutagenesis was performed following the basic proto-
cols from the QuickChange™ site-directed mutagenesis kit (Strat-
agene, La Jolla, CA). The following oligonucleotides were used for
the mutagenesis of serine 121: wild-type, 5'- C AT GTG GAC ACC TAT
TCC ATT GCA GCC GGG C 3'; Ser → Ala, 5'- C AT GTG GAC ACC
TAT CCT ATT GCA GCC GGG C 3; Ser → Cys, 5'- GC CAT GTG GAC
ACC TAT TAT GCT ATT GCA GCC GGG C 3; Ser → Thr, 5'- C AT GTG
GAC ACC TAT TAC ACG TAT GCA GCC GGG C 3' (bold characters
cite changed bases). Both sense and antisense complementary oli-
gonucleotides were used in the same PCR reaction, and the primers
were designed to introduce a second silent mutation that eliminated a
*SgrDI* site from the wild-type sequence to facilitate mutant screening.
50 ng of template DNA (BSOR gene in the GST vector); 125 ng each of
sense and antisense primer; 10% Triton X-100; 5% Me_{2}SO; 1.5 units of
*Pfu* DNA polymerase; dATP, dGTP, dCTP, and dTTP (each 200
mmol/L) and 1× *Pfu* buffer were mixed in a 100-μl reaction volume. Hot start
PCR was utilized for amplification of template DNA, with the mutation
introduced in both strands by the mutagenic oligonucleotide pair. 18
cycles of PCR corresponding to 95 °C for 30 s, 55 °C for 1 min, and 68 °C
for 1 min were performed following an initial denaturation step of
95 °C for 5 min. Following PCR, the wild-type dam methylated tem-
plate was digested with 10 units of *Dpn*I at 37 °C for 1 h. Following
template digestion, competent *E. coli* DH5a cells were directly trans-
formed with 2- and 4-μl aliquots of the digested PCR reaction without
further purification. Colonies that grew on the ampi-resistant LB plates
were grown and miniprepared for DNA isolation. Positive mutants were
identified following restriction digests with *SgrDI*, which in the mu-
tants cleaved the DNA at one less site than in the wild type. Following
initial screening, positive mutants were sequenced using the Sequenase
kit (U. S. Biochemical Corp.) and [α-^{35}S]dATP.

**Protein Expression and Purification**

For the isolation of both wild-type and mutant recombinant *R.
sphaeroides* BSORs, transformed *E. coli* JM109 cells were grown over-
night, and BSOR expression was induced by addition of isopropylthio-
galactoside as described previously (3). For BSOR isolation, the cells
were harvested by centrifugation at 9,000 × g for 20 min, and the cell
pellets were resuspended in phosphate-buffered saline supplemented
with dithiothreitol (10 mM), aprotinin (0.1 mg/ml), EDTA (1 mM), phen-
ylmethylsulfonyl fluoride (0.1 mM), and sodium molybdate (1 mM). The
cells were sonicated on ice, and the BSOR was purified as described
previously using a combination of glutathione-agarose affinity chroma-
tography, anion exchange chromatography (Mono-Q), and fast protein
liquid chromatography gel filtration (Superose 12) (3).

**Factor Xa Proteolysis**

Fusion protein samples were cleaved by treatment with Factor Xa
protease (1% w/v) in 50 mM Tris-HCl buffer containing 150 mM NaCl
and 1 mM CaCl_{2} (pH 8) at 16 °C for 16 h.

**Protein Analysis**

Recombinant wild-type and mutant BSORs were examined for purity
and size using SDS-PAGE. Protein samples (2–5 μg of total protein) were
analyzed using a 10.0% SDS-PAGE gel (15) stained with Coomas-
sie Blue.

**Enzyme Activities**

**Spectrophotometric assays—**BSOR activities (NADPH:BSOR and
NADPH:FR) were routinely determined using a Shimadzu (Columbia,
MD) UV2501 spectrophotometer. Assays were performed at 25 °C in 50
mM Tris-HCl buffer, pH 8.0 at 340 nm, monitoring the oxidation of
NADPH (250 μM) in the presence of either BSOR (1.7 mM) or FeCN_{6}^{3-}
(630 μM) as electron acceptor and using 2.5 μg of purified enzyme in a
final volume of 1 ml. NADPH concentrations were calculated using an
e_{340} of 6.22 mM^{-1} cm^{-1}. Activities, measured as initial rates, were
expressed as micromoles of NADPH consumed per minute per nano-
 mole of enzyme. Kinetic parameters were derived from the experimen-
tal initial rate data by least-squares fitting to the original hyperbolic
rate equation using the software Enzfitter (Elsevier Biosoft, Ferguson,
MO).

**Reverse Phase HPLC Analysis for Biotin Determinations—**Wild-
type and mutant BSOR activities were also examined using the
MV:BSOR assay and reverse phase HPLC to detect the formation of the
product, biotin. Each reaction contained 250 μl of 116 mM Mes, pH 6 buffer, 100
μl of 100 μM methyl viologen (in 50 mM Tris, pH 8), 100 μl of BSOR or
biotin (5 mg/ml in 20 mM NaOH), and 15 μg of enzyme. The mixture was
degassed for 1 h to eliminate any oxygen present, and the reaction was
initiated by injecting 20 μl of 1 mM dithionite. The reaction was allowed
to proceed for 0.5 h, following which the enzyme was separated from the
reactants and products by spin column filtration. 100 μl of the reaction
mixture was injected onto a C_{18} reverse phase column, and the reac-
tants were separated from the products using a gradient of 0.05% trifu-
oroacetic acid, pH 2.5 and 0.05% trifluoroacetic acid, acetonitrile
(70:30) as described previously (3).

**Molybdenum Cofactor Analysis—**Wild-type and mutant enzyme
samples were denatured with 1% SDS followed by boiling for 30 min. The
SDS was precipitated with KCl (0.25 M final concentration), and the
cofactor was separated from the protein utilizing ultrafiltration spin
columns (ultrafree-MC, 5,000 molecular weight cut-off, Millipore Corp.,
Bedford, MA). The presence of cofactor in each sample was determined
by fluorescence spectrosopy using a Shimadzu RF5301 spectropho-
umeter. Excitation spectra were obtained using an emission wavelength of
460 nm, and emission spectra were obtained using an excitation wave-
length of 370 nm. Isolated cofactor samples were treated with nucleo-
tide pyrophosphatase as described previously (3).

**Molybdenum Analysis—**Mo analysis was performed utilizing induct-
ively coupled plasma mass spectrometry at the University of Georgia,
Department of Chemistry, Athens, GA using sodium molybdate as
standard.

**Results**

**Wild-type and Mutant BSOR Expression and Purity—**To en-
sure that the kinetic properties of the wild-type and mutant
enzymes were not influenced by contaminating proteins, re-
combinant *R. sphaeroides* wild type and the three BSOR mu-
tants, corresponding to S121A, S121T, and S121C, were purified
to homogeneity and analyzed by SDS-PAGE. As shown in
Fig. 1, following anion exchange chromatography of the Factor
Xa-cleaved proteins, all of the purified enzymes exhibited a single,
high molecular mass band corresponding to a mass of
approximately 80 kDa following SDS-PAGE, which was of
the appropriate size predicted from the amino acid sequence.
An additional low molecular mass band of approximately
29 kDa was also apparent for each sample and corresponded to the
cleaved GST tag. All three mutant proteins were expressed as the
appropriately sized proteins even though the individual
expression efficiencies varied. Whereas the S121T mutant was
expressed at levels comparable with wild type, the expression
levels of the S121A and S121C mutants were approximately
2–5-fold lower than that of the wild-type BSOR.

Wild-type and Mutant BSOR Activities—To assess the functionality of the mutant proteins, their respective activities were examined utilizing the spectrophotometric assays for NADPH:BSOR and NADPH:FR activities and by HPLC detection of biotin produced following the MV:BSOR assay. In addition, all proteins were examined for their reactivity with alternative substrates, such as trimethylamine-N-oxide.

As illustrated in Table I, utilizing equal molar amounts of protein and compared with the wild-type enzyme, none of the mutants exhibited any significant, detectable activity using either the NADPH:BSOR or NADPH:FR assays. The very low levels of activity observed with some of the substrates, corresponding to 0.3–3% of wild-type values, were indistinguishable from background levels. In addition, there was no change in substrate specificity for any of the mutants examined.

The results of the reverse phase HPLC analysis of the MV$:BSOR activity reaction products of wild-type BSOR are shown in Fig. 2. Under the reaction conditions utilized for the assay, biotin eluted at 17.7 min and BSO eluted at 9.1 min, partially overlapping the peak at approximately 8.7 min resulting from the control reaction utilizing only methyl viologen, dithionite, and buffer (Fig. 2, trace D) in the absence of BSOR.

Biotin formation was detected only when the wild-type enzyme was utilized in the assay mixture (Fig. 2, trace C). In contrast, utilizing the S121A, S121T, or S121C mutants, no biotin production was observed in any of the assays (Fig. 2, traces E–G). Peak intensities observed following HPLC analyses of the wild-type and mutant reaction mixtures are shown in Table II, indicating the absence of any detectable conversion of BSO to biotin by the S121A, S121T, or S121C variants of BSOR.

Cofactor Analysis—The fluorescence excitation and emission spectra of the molybdenum cofactor isolated from wild-type BSOR and the S121A, S121T, and S121C mutants are shown in Fig. 3. All three mutant proteins were demonstrated to incorporate the Mo cofactor as shown by the fluorescence excitation and emission spectra, which yielded characteristic maxima at 370 and 470 nm, respectively, and were very similar to the corresponding spectra obtained from the wild-type enzyme. Small shifts toward lower wavelengths were observed for the fluorescence emission maxima, especially for the S121T and S121C mutants, which may reflect different oxidation states of the cofactor isolated from the mutant proteins. Treatment of the isolated cofactor samples with nucleotide pyrophosphatase resulted in the characteristic approximately two-fold increase in the fluorescence emission intensity, confirming the presence of the dinucleotide form of the molybdenum cofactor in all three mutants.

Molybdenum Analysis—Total Mo analyses of the wild-type, S121A, S121T, and S121C variants of BSOR were performed using inductively coupled plasma mass spectrometry. As shown in Table III, all the mutant proteins were determined to have incorporated Mo, although the mutants were observed to have slightly lower Mo stoichiometries when compared with the wild-type BSOR. The lowest level of Mo incorporation, corresponding to 72% of the wild-type BSOR Mo content, was observed for the S121T mutant, whereas Mo incorporation was higher for both the S121A and S121C mutants.

DISCUSSION

The preceding results represent the first application of site-directed mutagenesis to probe the role(s) of specific active-site amino acid residues in the function of R. sphaeroides BSOR.

| Activity                  | Wild type | S121T | S121A | S121C |
|---------------------------|-----------|-------|-------|-------|
| NADPH:BSOR                | 30.0      | 0.05  | 0.005 | 0.03  |
| NADPH:FR                  | 1.70      | 0.05  | 0.00  | 0.04  |
| NADPH:TMANOR              | 30.8      | 0.01  | 0.00  | 0.01  |
| NADPH:NNOR                | 19.8      | 0.06  | 0.003 | 0.01  |
| NADPH:ANOR                | 25.5      | 0.05  | 0.00  | 0.01  |
| NADPH:MSOR                | 6.10      | 0.00  | 0.00  | 0.00  |
| NADPH:DMSOR               | 9.60      | 0.00  | 0.00  | 0.00  |

* Specific activities were determined in 50 mM Tris buffer, pH 8 and are expressed as micromoles of NADPH consumed or BSO formed per minute per nanomole of BSOR.
Replacement of Ser<sup>121</sup> with Ala, Thr, or Cys residues has been demonstrated to result in the production of three mutant forms of BSOR that all retain the incorporation of both MGD and Mo but that are all devoid of both NADPH:BSOR and NADPH:FR activities.

Both BSOR and Me<sub>2</sub>SO reductase from <i>R. sphaeroides</i> share regions of extensive sequence similarity and exhibit the unique feature within the diverse array of known molybdoenzymes of containing MGD as their sole prosthetic group. Sequence alignments have indicated approximately 38% overall sequence conservation between the two proteins including regions of the primary structures identified as molybdenum cofactor-binding signatures (14).

X-ray crystallographic studies of Me<sub>2</sub>SO reductase have demonstrated the presence of a novel metal cluster comprising a single Mo and two MGD cofactors. The single Mo atom is coordinated by four thiols, two derived from each MGD cofactor. The single Mo atom is demonstrated to result in the production of three mutant forms of BSOR that all retain the incorporation of both MGD and Mo but that are all devoid of both NADPH:BSOR and NADPH:FR activities.

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X-ray crystallographic studies of Me<sub>2</sub>SO reductase have demonstrated the presence of a novel metal cluster comprising a single Mo and two MGD cofactors. The single Mo atom is coordinated by four thiols, two derived from each MGD cofactor, in addition to an oxygen atom (O<sub>2</sub>) that comprises part of the side chain of Ser<sup>147</sup> (12).

The results of multiple sequence alignments of members of the Me<sub>2</sub>SO reductase family of MGD-containing molybdoenzymes are shown in Fig. 4. In addition to Me<sub>2</sub>SO reductase from organisms such as <i>R. sphaeroides</i> (16) and <i>R. capsulatus</i> (17), the family also comprises BSOR from such organisms as <i>E. coli</i> (18), <i>Helicobacter pylori</i> (19), and <i>Haemophilus influenza</i> (20) and other <i>E. coli</i> enzymes, such as trimethylamine-N-oxide reductase (21), the dissimilatory forms of nitrate reductase (22, 23), and formate dehydrogenase (24, 25). Within the N-terminal portion of these sequences, the residue corresponding to Ser<sup>147</sup> in Me<sub>2</sub>SO reductase is strongly conserved as a serine, cysteine, or thiol (SeC) residue, indicating that in proteins belonging to the Me<sub>2</sub>SO reductase family of Mo-containing enzymes, the putative Mo ligand corresponds to either an oxo or sulfido group derived from an S, C, or SeC side chain.

The active site of BSOR has recently been examined using a combination of electron paramagnetic resonance, resonance Raman, and x-ray absorption spectroscopies (10, 11). The results of these studies indicate that BSOR has a very similar Mo site architecture to Me<sub>2</sub>SO reductase, with four thiol ligands donated by the two MGD cofactors and an oxo ligand donated by a serine residue, which has been proposed from sequence alignments to correspond to Ser<sup>121</sup>. A proposed catalytic scheme for BSOR, derived from the resonance Raman studies (11), has indicated that Ser<sup>121</sup> remains coordinated to the Mo center both in the oxidized and reduced states throughout the catalytic cycle of the enzyme. The results of our mutagenesis studies are in agreement with this active site model and suggest that the oxo group of Ser<sup>121</sup> provides the fifth ligand to the Mo center.

Recent site-directed mutagenesis studies of Ser<sup>147</sup> in Me<sub>2</sub>SO reductase have demonstrated that replacement of Ser<sup>147</sup> by Cys resulted in the production of a Me<sub>2</sub>SO reductase mutant that retained significant functional activity with both Me<sub>2</sub>SO and trimethylamine-N-oxide. Whereas substantial decreases in catalytic activities with alternate substrates such as methionine sulfoxide and BSO were observed, the S147C mutant was shown to exhibit enhanced activity (400% increase compared with wild type) with adenosine N<sup>6</sup>-oxide as the oxidizing substrate, suggesting a change in substrate specificity (13). Recent extended x-ray absorption fine structure studies (26) have also indicated that the side chain sulfur of the S147C variant functions as a molybdenum ligand, conferring partial activity.

In contrast, replacement of Ser<sup>21</sup> in BSOR with either Cys, Thr, or Ala resulted in the production of three nonfunctional mutants that were catalytically inactive when assayed spectrophotometrically with all of the oxidizing substrates utilized by the wild-type enzyme, suggesting that no alteration in substrate specificity occurred. In addition, HPLC analysis of the reaction products generated by the wild-type and mutant enzymes also clearly demonstrated that biotin formation was absent in the reactions catalyzed by all three mutants. We analyzed for product formation, rather than monitoring the disappearance of reduced methyl viologen, to avoid potential problems due to incomplete anaerobiosis during the assay. These results also confirmed that a functional Mo site is also required for both NADPH:BSOR and NADPH:FR activities, the latter representing a partial activity that is unique to <i>R. sphaeroides</i> BSOR.

Site-directed mutagenesis has also been utilized to examine...
the role of the putative Mo ligand in other members of the MGD-containing Me₂SO reductase family with varied results. Generation of the S176C mutant in the catalytic (dmsA) subunit of E. coli Me₂SO reductase resulted in the production of an inactive enzyme (27), whereas generation of the SerC141C mutant in E. coli formate dehydrogenase resulted in production of an enzyme with decreased activity (28). The variety of effects of residue substitution on the catalytic activities of members of the Me₂SO reductase family of enzymes suggests that the residue nature of the fifth ligand, derived from the protein side chain in the coordination environment of the Mo center, plays a pivotal role in regulating the functionality of the metal center. Changes in the coordination chemistry of the Mo center in these enzymes could have significant impact on the oxidation-reduction potentials of the Mo⁶⁺/Mo⁴⁺ and Mo⁴⁺/Mo⁶⁺ redox couples, could also alter the accessible redox states available during turnover or could change the conformation of the active site.

Our results indicate that Ser¹²¹ is an essential amino acid for functionality in BSOR. However, whereas this residue functions as a coordinating ligand to the Mo, it is not crucial for retention of either Mo coordination or MGD binding because all the mutants retained both cofactor and Mo. Additional spectroscopic studies of the Ser¹²¹ mutants will be required to determine whether the Cys or Thr variants fail to provide a suitable fifth ligand for molybdenum coordination, resulting in loss of activity.

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