An Active Site Tyrosine Influences the Ability of the Dimethyl Sulfoxide Reductase Family of Molybdopterin Enzymes to Reduce S-Oxides*

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Dimethyl sulfoxide reductase (DMSOR), trimethylamine-N-oxide reductase (TMAOR), and biotin sulfoxide reductase (BSOR) are members of a class of bacterial oxiotransferases that contain the bis(molybdopterin guanine dinucleotide)molybdenum cofactor. The presence of a Tyr residue in the active site of DMSOR and BSOR that is missing in TMAOR has been implicated in the inability of TMAOR, unlike DMSOR and BSOR, to utilize S-oxides. To test this hypothesis, Escherichia coli TMAOR was cloned and expressed at high levels, and site-directed mutagenesis was utilized to generate the Tyr→Ala and Phe variants of Rhodobacter sphaeroides DMSOR and insert a Tyr residue into the equivalent position in TMAOR. Although all of the mutants turn over in a manner similar to their respective wild-type enzymes, mutation of Tyr-114 in DMSOR results in a decreased specificity for S-oxides and an increased specificity for trimethylamine-N-oxide (Me₃NO), with a greater change observed for DMSOR-Y114A. Insertion of a Tyr into TMAOR results in a decreased preference for Me₃NO relative to dimethyl sulfoxide. Kinetic analysis and UV-visible absorption spectra indicate that the ability of DMSOR to be reduced by dimethyl sulfide is lost upon mutation of Tyr-114 and that TMAOR does not exhibit this activity even in the Tyr insertion mutant.

Rhodobacter sphaeroides and Rhodobacter capsulatus dimethyl sulfoxide reductase (DMSOR),† Escherichia coli and Shewanella massilia trimethylamine-N-oxide reductase (TMAOR), and R. sphaeroides biotin sulfoxide reductase (BSOR) are members of a class of bacterial oxiotransferases that all contain the bis(molybdopterin guanine dinucleotide) form of the molybdenum cofactor (bis(MGD)Mo) seen in Fig. 1 (1–4). These enzymes are ideal targets for spectroscopic and kinetic studies of the molybdenum center since they do not contain additional cofactors. In contrast, in all other molybdoproteins studied to date, the low energy absorption bands of the molybdenum atom are overshadowed by prosthetic groups such as hemes, iron-sulfur centers, and flavins. Mechanistic studies are aided by the extensive structural information reported for this family, including several x-ray crystal structures (2, 4–8), analysis by extended x-ray absorption fine structure spectroscopy (EXAFS) (9–11), EPR (10–14), and resonance Raman spectroscopy (15, 16).

Resonance Raman studies on BSOR and DMSOR indicate that both enzymes function by an oxo transfer mechanism whereby the oxo group from the substrate is directly transferred to the molybdenum atom (Fig. 2) (15, 16). This process is reversible in DMSOR, and the ability of this enzyme to be reduced by dimethyl sulfide (Me₂S) has been extensively studied (7, 11, 15, 17). However, resonance Raman analysis has indicated that BSOR is unable to be reduced with either Me₂S or by biotin (16). Other than mutation of the protein ligands to the molybdenum in DMSOR and BSOR (18, 19), no mutagenic studies have been reported for TMAOR, BSOR, or Rhodobacter DMSOR, and there is little information about the roles of other amino acids in enzymatic activity.

Alignment of R. sphaeroides and R. capsulatus DMSOR to E. coli and S. massilia TMAOR and R. sphaeroides BSOR indicates 22% sequence identity and 48% sequence similarity between all five enzymes. Despite this similarity, there are striking differences in their physiological roles, electron donors, and substrate specificity. TMAOR functions as the final enzyme in the anaerobic electron transport pathway that utilizes Me₃NO as the terminal electron acceptor (20). Although DMSOR also functions as a terminal enzyme during anaerobic respiration, it is able to utilize a greater variety of substrates including Me₃NO and dimethyl sulfoxide (Me₃SO) (21). BSOR, a cytoplasmic protein whose possible roles include scavenging biotin sulfoxide (BSO) to generate biotin and protecting the cell from oxidative damage (22), has also been shown to use a variety of S- and N-oxides (23).

The x-ray crystallographic structure of DMSOR has generated great interest in the role of the Tyr at position 114 during catalytic turnover of the enzyme. In the 1.3-Å crystal structure of R. sphaeroides DMSOR, the bis(MGD)Mo active site exhibits two different coordination geometries (5). In the catalytically active, hexa-coordinated molybdenum site, the single oxo group is coordinated by Trp-116 (Fig. 3B), whereas in the inactive, penta-coordinated molybdenum environment, Tyr-114 is hydrogen-bonded to one of two oxo groups (Fig. 3C). Although sequence alignments have shown that BSOR contains a residue equivalent to Tyr-114, this residue is missing in E. coli and S. massilia TMAOR (Fig. 3, A and D). Since both BSOR and DMSOR are able to reduce a wide variety of S- and N-oxides whereas TMAOR shows a more limited specificity for N-oxides (24), Tyr-114 has been postulated to be responsible for this variance in substrate specificity (4, 25).
Role of Tyr-114 in Substrate Specificity of R. sphaeroides DMSOR

Although R. sphaeroides DMSOR and BSOR were previously cloned and heterologously expressed in E. coli (3, 23, 26), TMAOR has not been cloned previously. In the studies reported here, E. coli TMAOR has been cloned and the recombinant protein purified, setting the stage for a comprehensive study of the role of Tyr-114 in this family of enzymes. This residue has been mutated to both Ala and Phe in DMSOR, and a Tyr has been inserted into TMAOR in an equivalent position. The molebdenum coordination environment of the wild-type and mutant proteins has been probed using UV-visible absorption spectroscopy. The activities of both wild-type and mutant proteins have been analyzed by steady-state kinetics with both S- and N-oxides in the forward direction, and the efficiency of Me2S reduction of these enzymes has been measured. The presence of these mutations does not appear to affect stability or cofactor incorporation. The mutation of Tyr-114 in DMSOR does increase the specificity for N-oxides while decreasing the specificity for S-oxides, and insertion of the Tyr residue in TMAOR increases the specificity for S-oxides with a concomitant decrease in specificity for N-oxides. Mutation of Tyr-114 in DMSOR also results in inefficient reduction of the enzyme by Me2S.

EXPERIMENTAL PROCEDURES

Cloning of E. coli TMAOR—The first 117 nucleotides of the E. coli torA sequence encode a 39-amino acid N-terminal signal sequence that targets TMAOR to the periplasm and is cleaved to form the mature enzyme (27). The structural gene for TMAOR without the periplasmic signal sequence was isolated from DH5α genomic DNA using PCR primers created from the E. coli K12 torA gene sequence (GenBank™ accession number X73588). One primer inserted an NdeI site and a new start codon immediately 5′ to the codon for the first amino acid of the mature protein, and the second created a HindIII site 36 nucleotides downstream from the stop codon. The resulting DNA segment containing the torA sequence was ligated directly into the pCR2.1 cloning vector (Invitrogen). The consensus sequence of three independent clones confirmed the previously published K12 sequence (27). Primers were obtained from Life Technologies, Inc., and automated sequencing was accomplished by the Duke University DNA Analysis Facility.

Creation of TMAOR Expression Construct—One plasmid clone containing a single polymerase error that changed Trp-576 to Ala was selected for further manipulations. Due to difficulties in growing cells containing the pCR2.1 cloning vector during mutagenesis, the TMAOR coding sequence was released with HindIII and transferred into the pBluescript II Ks(+) cloning vector (Stratagene). The polymerase error was repaired by site-directed mutagenesis on double-stranded DNA using the CLONTECH Transformer Site-directed Mutagenesis Kit to form pKJ125. This plasmid was digested with NdeI and HindIII, and the coding sequence was ligated into the pET-28a(+) expression vector (Novagen) to form pKJ525 (Table I) which encodes for mature TMAOR. To aid in purification of the protein, the TMAOR coding region of pKJ125 was released with HindIII and NdeI and ligated into pET-28a(+) (Novagen) to form pKJ725, which encodes an N-terminal His6-tagged version of TMAOR. The coding sequence containing the structural gene, and the N-terminal His tag from pKJ725 was excised using HindIII and NcoI and ligated into pTrc99 A to form pKJ825. This plasmid expresses the TMAOR+Y variant of TMAOR, which contains a Tyr residue in a location equivalent to Tyr-114 in DMSOR.

Creation of TMAOR Constructs—Site-directed mutagenesis was performed on pHJ118 (18) to change the Tyr at position 144 to either Ala or Phe. The mutated coding sequences were subsequently liberated using HindIII and NdeI and ligated into pTrc99 A to form pKJ830. This plasmid expresses the TMAOR+Y variant of TMAOR, which contains a Tyr residue in a location equivalent to Tyr-114 in DMSOR.

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Expression—All of the pTrc 99 A-based expression vectors (pJH820 (28), pKJ825, and pKJ830) were transformed into RK4353 E. coli cells (29). Growth and expression in these cells were done as described previously (3) with the exception that isopropyl-β-D-thiogalactopyranoside (IPTG) was present at 10 μM. The RK4353 E. coli strain was DE3-lysogenized using the λ DE3 lysogenization kit from Novagen to create the RK4353(DE3) strain. All of the pET-based expression plasmids (pJH720, pKJ725, pKJ525, pJN711, and pJN712) were transformed into these cells. The resulting strains were subsequently transformed a second time with pLysS (Novagen). Conditions for growth and expression were as described previously (3), with the exception that 30 μg/ml kanamycin and 1 μg/ml chloramphenicol were used as the sole antibiotics, and induction was achieved by three passages through the 112-μm lysis filter. The lysis was achieved by three passages through the 112-μm lysis filter. The lysozyme was activated by incubation at 37 °C for 25 min at 4 °C. The supernatant was then equilibrated with 40 ml of Ni2+-NTA affinity resin (Qiagen) by gentle stirring at 4 °C for 15 min. This slurry was loaded onto a gravity flow column and subsequently washed with 2 column volumes of PN buffer at pH 7.5 containing 10 mM imidazole, 8 column volumes of the same solution at pH 8.0, and 3 column volumes of PN buffer, pH 8, containing 20 mM imidazole. TMAOR was eluted at pH 8.0 with PN buffer containing 200 mM imidazole. The fractions containing TMAOR were combined, dialyzed against 50 mM Tris, pH 7.5, and purified using a Q-Sepharose fast protein liquid chromatography column with a 23-ml bed volume. The protein was resolved using a linear gradient of 0–250 mM NaCl, followed by a 2-column volume 0–300 mM gradient of NaCl, and 0.4 mM 2,6-dichlorophenolindophenol (DCPIP). Background activity, obtained by the addition of anaerobic MeS to a cuvette containing no enzyme, was subtracted to obtain all final activity numbers. All substrates were obtained from Sigma.

RESULTS

Creation of Expression Constructs—Whereas E. coli TMAOR has previously been purified from source (30, 31) and a similar enzyme has been purified from S. massilia (32), the yield in both cases was substantially less than that obtained by the heterologous expression of R. sphaeroides DMSOR and BSOR in E. coli (3, 28). To facilitate purification of the large quantities of enzyme required for comprehensive studies on the role of the Tyr residue in DMSOR and TMAOR, E. coli TMAOR was cloned, homogeneously expressed, and purified. Overexpression, the gene for mature E. coli TMAOR with an added N-terminal His6 tag was placed into the pTrc 99 A vector. Three mutants were created to elucidate the role of Tyr-114. In DMSOR, this residue was mutated to either Ala or Phe. The latter retains the large phenyl ring but cannot form a hydrogen bond to the molybdenum oxo ligand. The mutated sequences were transferred into a vector to generate His-tagged versions of DMSOR-Y114A and DMSOR-Y114F. In TMAOR, a Tyr codon was inserted into the His-tagged coding sequence between residues 119 and 120 in a position corresponding to Tyr-114 of DMSOR (Fig. 3A).

Expression Conditions—BL21(DE3), the standard E. coli strain used for expression of pET plasmids, appears to have difficulty expressing enzymes containing the molybdenum cofactor (33). RK4353 is the base strain for an extensive series of cofactor biosynthesis mu insertion mutants (29), and it was chosen as an alternative expression strain. Since expression from pET-based plasmids requires the presence of T7 polymerase, the RK4353 strain was DE3-lysogenized. The plasmids expressing TMAOR reductase with and without a His tag (pKJ725 and pKJ725, DMSOR (pJH720), DMSOR-Y114A (pJN711), and DMSOR-Y114F (pJN712) were transformed into RK4353(DE3) cells. The resulting strains were subsequently transformed with pLysS.

Because of problems with cell stability as a result of DE3 lysogenization, an alternative expression system was created. Expression of R. sphaeroides DMSOR in the pTrc 99 A expression construct has been shown to produce a substantial increase in yield and permits expression in an alternative cell line, S. cerevisiae, without the presence of T7 polymerase (28). The plasmids expressing His-tagged TMAOR, TMAOR-Y, and DMSOR in pTrc 99 A (pKJ825, pKJ830, and pJH820, respectively) were transformed into RK4353 E. coli cells.

All strains were grown and induced for 24 h under anaerobic conditions as described previously (3) with the exception that kanamycin and chloramphenicol were used as antibiotics for all
RK4353(DE3) pLysS strains containing pET-based plasmids, and ampicillin was used for all expression from RK4353 cells containing pTet 99 A-based plasmids. The IPTG concentration had to be lowered to 10 μM for expression of proteins from the pTet 99 A vectors to obtain a level of active protein equivalent to that expressed from the pET vectors when induced with 40 μM IPTG.

**Purification of TMAOR—**Analysis of DMSOR has shown that the presence of a N-terminal His tag does not alter the activity of the protein (18). To ascertain whether the same was true for TMAOR, both the native and His-tagged versions of the protein were expressed in RK4353(DE3) cells. After partial purification, the proteins exhibited similar k_cat and K_m values with Me_3NO, indicating that the His tag does not interfere with catalytic competence. The elution profiles of both proteins on a GF-250 size exclusion high pressure liquid chromatography column were also similar, indicating equivalent folding and subunit composition. Therefore, tagged TMAOR was used for all further studies reported here.

TMAOR was purified to greater than 95% homogeneity after a 65 °C heat step was followed by Ni²⁺-NTA affinity and Q-Sepharose ion-exchange chromatography, as seen in Table II. Previous work with DMSOR has shown that purified, recombinant protein displays an altered absorption spectrum (18) and molybdenum coordination environment (11) compared with protein purified from source or recombinant protein after catalytic turnover. As shown in Fig. 4, absorption spectroscopy analysis indicates a similar phenomenon with TMAOR. The TMAOR Mo(VI) spectra obtained after purification without catalytic turnover is different from the Mo(VI) spectra obtained after one or more rounds of reduction and substrate oxidation. The uncycled TMAOR is unstable and shows rapid loss of activity at 4 °C associated with loss of the molybdenum atom. Activity assays with this species exhibit a distinct lag before a linear rate is observed. Upon addition of sodium dithionite, a light green color. The reduced Mo(IV) spectra differ very little among all five species with the main absorption peak exhibit-...
TABLE III
Expression levels and molybdenum incorporation in wild-type and mutant TMAOR and DMSOR

| Molybdenum | mg/liter |
|------------|---------|
| DMSOR      | 65      | 5.1   |
| DMSOR-Y114A| 61      | 3.2   |
| DMSOR-Y114F| 62      | 2.7   |
| TMAOR      | 66      | 2.1   |
| TMAOR+Y    | 47      | 3.5   |

*% protein/μm molybdenum. Protein concentration was determined using ε = 197,000 m^−1 cm^−1, and molybdenum concentration was determined using atomic absorption spectroscopy.

**mg protein purified/liter of cell growth.**

determined for purified enzyme and normalized to 100% molybdenum content. The mutations appear to alter the specificity for both S- and N-oxides. DMSOR-Y114F shows a significant decrease in the k_{cat}/K_m for both S-oxides and a small increase in the k_{cat}/K_m for MeNO. Whereas DMSOR-Y114A shows a similar decrease in k_{cat}/K_m for Me2SO and MetSO to DMSOR-Y114F, there is a much larger increase in the k_{cat}/K_m for MeNO. In TMAOR+Y, the k_{cat}/K_m for Me2SO increases slightly, and the k_{cat}/K_m for MeNO decreases greatly.

Although the dominant effect of these mutations is on the K_m changes, in the k_{cat} values also influence the specificity. Both mutations in DMSOR show 2 orders of magnitude increases in the K_m for Me2SO and MetSO relative to DMSOR. However, DMSOR-Y114F also shows a greater increase in the k_{cat} values and, therefore, retains specificity constants closer to that of the wild-type enzyme. Similarly, this explains why little change is seen in the specificity constant for ANO. Whereas both mutations in DMSOR cause an order of magnitude increase in the k_{cat}, this is matched by a similar order of magnitude increase in the K_m, and the specificity constant changes no more than 2-fold.

Comparison of specificity constants (Table V) shows that DMSOR prefers the S-oxides, Me2SO and MetSO, to MeNO by a factor of 210 and 5.3, respectively, and TMAOR prefers MeNO to Me2SO and MetSO by a factor of 5,600 and 2,100, respectively. Mutation of Tyr-114 to a Phe in DMSOR significantly decreases the preference for MeNO, and MeNO is actually preferred to MetSO by a factor of 5. Mutation to an Ala changes the specificity more dramatically such that DMSOR-Y114A shows a greater preference for MeNO than for either S-oxide. The opposite effect is seen upon the addition of a Tyr to TMAOR where there is a marked decrease in the preference for MeNO. TMAOR+Y prefers MeNO to Me2SO by a factor of 100 rather than the factor of 5,600 seen for the wild-type enzyme. TMAOR+Y also prefers MeNO to MetSO by a factor of 150, an order of magnitude less than that seen for wild-type TMAOR.

**Reduction with Me2S—** Whereas the ability of DMSOR to be reduced by Me2S has been studied extensively using various techniques (11, 15, 17), BSOR cannot be reduced by either Me2S or biotin (16), and the activity of TMAOR upon product addition has not been studied previously. To investigate how mutation of Tyr-114 alters this activity, data were obtained as described by Adams et al. (17) using PMS and DCPIP as electron acceptors (Table VI). Attempts were also made to obtain the Me2S-reduced UV-visible absorption spectra with all seven enzymes (Fig. 6).

Although DMSOR purified from R. sphaeroides showed the expected activity in the reverse assay, recombinant DMSOR, DMSOR-Y114F, and DMSOR-Y114A showed no activity unless they had been first cycled and dialyzed before the assays were performed. The values for DMSOR purified from R. sphaeroides and recycled, recombinant DMSOR (6.5 and 8.2 s^{-1}), respectively, were comparable to the previously published activity of R. capsulatus DMSOR of 8 s^{-1} (17). The activity of wild-type TMAOR was 0.0035 s^{-1}, 3 orders of magnitude less than for DMSOR. Both mutations in DMSOR showed a shift in activity toward that seen for TMAOR. The activity of DMSOR-Y114F (0.3 s^{-1}) was an order of magnitude less than DMSOR, whereas that for DMSOR-Y114A was 2 orders of magnitude less (0.06 s^{-1}). Significantly, the introduction of a Tyr residue into TMAOR resulted in an increase in the V_max from 0.0035 to 0.13 s^{-1}, to a level comparable to that of DMSOR-Y114F. The K_m values, however, were not very different between TMAOR and TMAOR+Y, being 80 and 60 mM, respectively.

The Me2S-reduced spectra of both DMSOR purified from R. sphaeroides (Fig. 6A) and the recombinant protein after cycling (Fig. 6B) are very similar to that published for the R. capsulatus enzyme (17). TMAOR shows very little activity in the PMS/DCPIP assay upon addition of Me2S or Me3N, and the (Mo(VI)) spectrum does not change with the addition of 200 mM Me2S or 200 mM Me3N. Although the reverse assay indicates activity for DMSOR-Y114F, the absorption spectrum does not show any significant reduction by Me2S (Fig. 6C). Although the spectrum does change upon addition of 50 mM Me2S, this change does not appear to be caused by reduction of the enzyme. There is no color change as seen in wild-type DMSOR, and the decrease in absorbance in the 720 peak is not as large as seen in the wild-type reduced enzyme, and the addition of Me2S does not reverse the changes. DMSOR-Y114A (Fig. 6D) and TMAOR+Y (Fig. 6E) also exhibit slight spectral changes upon Me2S addition that do not appear to be caused by reduction of the enzymes.

**DISCUSSION**

Although the differences seen in the three different crystal structures of DMSOR have been recently resolved (5), the obvious flexibility of its active site still encourages comparison to similar enzymes. Whereas E. coli and S. massilia TMAOR have been purified previously from source, this is the first time that E. coli TMAOR has been cloned and overexpressed. Together with R. sphaeroides DMSOR and BSOR, this is the third member of the DMSOR family of molybdopterin enzymes to be cloned and purified at high levels in this laboratory. Overexpression consistently produces 1.3 mg of active protein/liter (0.3 mg of active protein/μg cell), whereas purification of native enzyme has only provided, at best, 0.05 mg of protein/μg cells (32). This provides a good system for mutagenic studies designed to investigate the mechanism of the DMSOR family and for detailed comprehensive studies using techniques such as EXAFS, resonance Raman, and EPR.

Mutation of the Tyr-114 in DMSOR and insertion of an equivalent Tyr into TMAOR does not significantly alter the stability of the proteins or interfere with cofactor or molybdenum binding. Molybdenum incorporation is comparable to wild type, and none of the mutants exhibit the heat instability exhibited by DMSOR lacking the complete cofactor (28). UV-visible absorption spectra of oxidized and reduced forms indicate that both DMSOR Tyr-114 mutations and TMAOR+Y turn over in a manner similar to their respective wild-type proteins. Although there are some shifts in the cyclic Mo(VI) absorption spectra of the mutants at longer wavelengths, all of the main features seen for the wild-type proteins still remain, indicating that the molybdenum coordination environments are similar. Resonance Raman studies have indicated that the absorption peak at 550 nm is associated with the Mo—O—bond bond found in the hexa-coordinate, mono-oxo spectra (15, 16). This absorption peak is changed very little from wild type in either of the Tyr mutants, and it does not appear that the presence or absence of this Tyr greatly perturbs the catalytic Mo—oxygen
Role of Tyr-114 in Substrate Specificity of R. sphaeroides DMSOR

bond. The as purified, oxidized spectra of all recombinant proteins differ from the oxidized spectra obtained after reduction with dithionite and re-oxidation with the appropriate substrate. Whereas the Mo(IV) and cycled Mo(VI) spectra of TMAOR are very similar to the same spectra of DMSOR and BSOR, the uncycled form of TMAOR exhibits several characteristics that differ from as purified DMSOR. EXAFS analysis of as purified, recombinant DMSOR indicates that, whereas all four dithiolene sulfurs are ligated to the molybdenum, the serine ligand is not bound until after the protein has been reduced with dithionite (11). Investigation of uncycled TMAOR by resonance Raman and EXAFS should provide new information about another alternative active site conformation. Although EXAFS, resonance Raman, and crystallography have supported the existence of a mono-oxo Mo(VI) to des-oxo Mo(IV) redox cycle (5, 10, 11, 15, 16), the flexibility of the

![Image](https://www.jbc.org/)

**TABLE IV**

Kinetic parameters with a variety of substrates

All numbers were determined as described under "Experimental Procedures" in 50 mM Tris, pH 7.5, and 0.15 mM methyl viologen. DMSOR was present at 0.45–2.4 μg/ml with Me₂SO as substrate, 1.2–4 μg/ml with MetSO, 0.03–0.35 μg/ml with MetNO, and 0.5 μg/ml with ANO. Y114F was present at 0.35–2.8 μg/ml with Me₂SO, 0.35–2.8 μg/ml with MetSO, 0.02–0.25 μg/ml with Me₃NO, and 0.04–0.1 μg/ml with ANO. Y114A was present at 0.65–3.8 μg/ml with Me₂SO, 0.8–6.3 μg/ml with MetSO, 0.05–0.25 μg/ml with Me₃NO, and 0.1–1.6 μg/ml with ANO. TMAOR concentrations were 4–9 μg/ml with Me₂SO as substrate, 1.2–8 μg/ml with MetSO, 0.035 μg/ml with Me₃NO, and 0.1–0.4 μg/ml with ANO. TMAOR+Y concentrations were 8.5–26 μg/ml with Me₂SO, 6.5 μg/ml with MetSO, 0.1 μg/ml with Me₃NO, and 0.2 μg/ml with ANO.

![UV-visible absorption spectra of DMSOR](https://www.jbc.org/)

**FIG. 5.** UV-visible absorption spectra of DMSOR (——), DMSOR-Y114F (—–), DMSOR-Y114A (—-), TMAOR (——), and TMAOR+Y (——). A, Mo(IV) spectrum obtained by dithionite reduction under anaerobic conditions. B, Mo(VI) spectrum obtained under anaerobic conditions after dithionite reduction and oxidation with Me₂SO (DMSOR, Y114A, and Y114F) or Me₃NO (TMAOR, TM+Y). All spectra were normalized to 5 mg/ml and 100% molybdenum.

![Table V](https://www.jbc.org/)

**TABLE V**

Substrate specificity relative to Me₃NO

![Diagram](https://www.jbc.org/)

Mo(IV) spectra Mo(VI) spectra

![Graph](https://www.jbc.org/)

![Graph](https://www.jbc.org/)
Mutation of Tyr-114 to Phe and Ala results in a decreased efficiency for reduction of Me$_3$NO. Whereas DMSOR-Y114F is already indicated that this enzyme does not efficiently reduce S-oxides (24), and the data presented here shows that insertion of a Tyr residue into this enzyme results in an increased ability to reduce S-oxides relative to Me$_3$NO. Although the Y114A mutation in DMSOR was not analyzed, in light of the comparison of TMAOR with and without a residue at this position, it is assumed that deletion of this residue in DMSOR would show the same shift to a preference for N-oxides that was observed in the two Tyr-114 DMSOR mutants.

E. coli also contains a DMSOR that is able to use Me$_3$NO under physiological conditions. Although the molybdnum-containing subunit of E. coli DMSOR shows homology to \textit{Rhodobacter} DMSOR and TMAOR, this enzyme also contains an electron transfer subunit containing four [4Fe-4S] clusters and a membrane anchor subunit (36). This enzyme is able to use both Me$_3$NO and Me$_2$SO under physiological conditions and shows kinetic values similar to those of \textit{Rhodobacter} DMSOR, with $k_{cat}/K_m = 1.9 \times 10^8 \text{M}^{-1} \text{s}^{-1}$ for Me$_3$NO (37) and $k_{cat}/K_m = 3.3 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ for Me$_2$SO (21). Although \textit{Rhodobacter} and \textit{E. coli} DMSOR are able to efficiently use Me$_3$NO as a physiological substrate, they do not catalyze the reaction as efficiently as TMAOR. The mutational analysis in this paper has shown that an increased efficiency in reduction of Me$_3$NO can be accompanied by a decreased efficiency in Me$_2$SO reduction and vice versa. This may explain the need for a separate TMAOR in \textit{E. coli} since it allows maximization of the respiration efficiency with Me$_3$NO without compromising respiration on Me$_2$SO.

Significant differences are seen in the ability of these enzymes to proceed in the reverse direction. Although DMSOR is reducible by Me$_2$S but not by Me$_3$NO, previous work (16) with BSOR has shown that it cannot be reduced with Me$_3$S, Me$_2$N, or biotin. It has now been shown that TMAOR is not reduced upon the addition of either Me$_2$S or Me$_2$N. The as purified, recombinant DMSOR exists in a di-oxo, non-catalytically active form that does not have the serine ligand to the molybdynium (11). Although dithionite reduction is sufficient to attach the serine ligand and to convert the protein to the catalytically active form, Me$_3$S is not strong enough to reduce the uncycled protein.

The presence of the Tyr residue near the cofactor appears to be essential but not sufficient for sustaining reduction by Me$_3$S in this family of enzymes. The $V_{max}$ is reduced by an order of magnitude for DMSOR-Y114F and 2 orders of magnitude for DMSOR-Y114A when monitored using the Me$_2$S/PMS/DCPIP assay. Insertion of a Tyr into TMAOR also increases the catalytic efficiency for reduction with Me$_2$S but has no effect on the $K_m$. Although a Me$_2$S-reduced absorption spectrum can be obtained for \textit{R. sphaeroides} DMSOR that is very similar to that already published for the \textit{R. capsulatus} enzyme (17), addition of Me$_2$S to DMSOR-Y114F and DMSOR-Y114A does not appear to result in the spectra of the reduced enzymes. Resonance Raman analysis of BSOR indicates that whereas the enzyme cannot be reduced by biotin or Me$_3$S, both appear to bind to the oxidized enzyme and alter the resonance Raman spectra (16). Therefore, it is likely that the changes seen in the UV-visible spectra for DMSOR-Y114F, DMSOR-Y114A, and TMAOR+Y upon Me$_3$S addition result from a Me$_3$S-bound form of the oxidized enzyme. Although studying the Me$_2$S-reduced form of DMSOR has provided valuable insight into the mechanism of this family, it does have limitations. It is unclear what physiological repercussions result from the loss of the Me$_3$S oxidase activity, since neither BSOR nor TMAOR can be reduced by their substrates. The availability of the physiological electron...
Role of Tyr-114 in Substrate Specificity of R. sphaeroides DMSOR

A reduced activity for Me3NO, analytical metal analysis was reductase activity. Although this enzyme also appears to show changes, including a loss in ability to be reduced by Me2S (38).

R. capsulatus derivative of data were presented for activity with Me3NO, the tungsten containing TMAOR, the value of 0.006 (25) is comparable to the substrate specificity have been reported for fact that this residue is close to the molybdenum atom and may likely explanation to both of these questions arises from the sufficient on its own to modulate that specificity. The most significant for this class of enzymes cannot be answered without more complete mechanistic studies. Although Tyr-114 has an impact on its own to modulate that specificity. The most significant for this class of enzymes cannot be answered without more complete mechanistic studies. Although Tyr-114 has an impact on its own to modulate that specificity. The most significant for this class of enzymes cannot be answered without more complete mechanistic studies. Although Tyr-114 has an impact on its own to modulate that specificity. The most significant for this class of enzymes cannot be answered without more complete mechanistic studies.

In all three of these cases, the change in specificity is matched by a change in redox potential. Detailed resonance Raman, EXAFS, EPR, and x-ray crystallographic studies on the mutants generated in these studies should provide greater understanding of how alterations in the vicinity of the molybdenum atom affect the catalytic properties of the enzyme.

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