Consequences of Removal of a Molybdenum Ligand (DmsA-Ser-176) of Escherichia coli Dimethyl Sulfoxide Reductase*

(Received for publication, February 5, 1996, and in revised form, August 7, 1996)

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We have used site-directed mutagenesis and EPR spectroscopy to examine the consequences of altering the molybdenum ligand in Escherichia coli dimethyl sulfoxide (Me₂SO) reductase (DmsABC). Mutagenesis of DmsA-Ser-176 to Ala, Cys, or His abolishes both respiratory growth on Me₂SO and in vitro benzyl viologen: Me₂SO oxidoreductase activity. EPR spectroscopy reveals changes in the line shape and the gₐ of the Mo(V) signals of the S176A and S176C enzymes. The midpoint potentials (Eₘ₋ₐ) of the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples in DmsABC are -15 and -175 mV. The Eₘ₋ₐ of the Mo(V)/Mo(IV) couple in the S176A mutant is 35 mV; however, the Mo(V) species could not be further oxidized with ferricyanide. Titration of the S176C mutant produced several overlapping Mo(V) species occurring at Eₘ > -150 mV, suggesting heterogeneity in the molybdenum environment. A Mo(V) signal was not visible in S176H membranes poised between -435 to 350 mV or oxidized with 200 μM ferricyanide. No differences were detected in the EPR spectra of the reduced [4Fe-4S] clusters of DmsABC and the S176A and S176H mutant enzymes; however, the S176C mutation altered the EPR line shape of one of the reduced [4Fe-4S] clusters.

Dimethyl sulfoxide (Me₂SO) reductase (DmsABC) of Escherichia coli is a membrane-bound terminal reductase that supports anaerobic respiratory growth on Me₂SO (1, 2). It is a complex molybdenum-molybdopterin guanine dinucleotide (Mo-MGD) cofactor and [Fe-S] cluster-containing enzyme located on the cytoplasmic surface of the plasma membrane (3, 4). DmsA (87.4 kDa) is the largest subunit and binds Mo-MGD at its active site (5). DmsB (23.1 kDa) is the electron transfer subunit and ligates four [4Fe-4S] clusters (6–9). DmsC (30.8 kDa) is an integral membrane protein that anchors the DmsAB subunits to the membrane and is necessary for menaquinol oxidation (9, 10). DmsABC belongs to a family of molybdenum-containing oxidoreductase enzymes with highly conserved organization and sequence (1, 11–13). These are prokaryotic enzymes that reduce Me₂SO (11, 14, 15), trimethylamine N-oxide (16), nitrate (17–19, 21, 24, 25), 2–4 biotin sulfoxide (14, 26, 27), polysulfide (29), and thiosulfate (30) or oxidize formate (31–35). These enzymes all bind molybdenum cofactor, specifically Mo-MGD, in the enzymes that have been characterized (5, 36). The cofactor from one of the enzymes in this family, the periplasmic M₇₆SO reductase (DSMR) from Rhodobacter sphaeroides, has been shown to be a bis-(MGD)-Mo (37), and the crystal structure of this enzyme has also recently been reported (38).

There are several forms of molybdenum cofactors that all consist of a molybdenum atom (or a tungsten in some cases) associated with molybdopterin mono- or dinucleotide (36). The molybdenum is proposed to be coordinated by the two di(thiolene) sulfur atoms of the molybdopterin (36), and this coordination of the molybdenum has been confirmed by the x-ray structures of three molybdopterin cofactor-containing enzymes (38–40). Pyrococcus furiosus aldehyde ferredoxin oxidoreductase contains a bis-molybdopterin-tungsten cofactor in which the tungsten is bound by the four di(thiolene) sulfur atoms of the molybdopterins, and an additional two coordination sites are occupied by oxo groups, glycerol from the buffer, or both (39). No ligands of the tungsten are from the protein. Aldehyde oxidoreductase from Desulfovibrio gigas contains a molybdopterin cytidine dinucleotide-Mo cofactor (40). The molybdenum is five coordinate with two sites occupied by the di(thiolene) sulfur atoms and three oxygen ligands. Two of the oxygen ligands are presumably oxo groups, and the third may come from the protein (40).

In the crystal structure of the Rh. sphaeroides DMSR (38), molybdogenium ligation is provided by the four di(thiolene) sulfur atoms of the molybdopterins, an oxo group, and a Ser residue (Ser-147).

The structure of Rh. sphaeroides DMSR is the most closely related to E. coli DmsA. DMSR is a soluble, periplasmically localized enzyme that receives reducing equivalents from periplasmically localized c-type cytochromes rather than from other subunits of a membrane-bound heterotrimer. However, comparison of the sequence of the E. coli DmsA subunit and the Rh. sphaeroides enzyme suggests a structural and functional similarity (54.4% similarity and 32% identity) between the two enzymes. In DMSR, the direct ligand to the molybdenum (Ser-147) is located in a highly conserved region of protein sequence within the family of bacterial molybdooenzymes that reduce S- and N-oxides (Fig. 1) (13, 42). Previous EXAFS studies of DMSR suggested that the molybdogenium ligation sphere included four sulfur atoms, presumably from two MGD, one oxo ligand, and one oxygen or nitrogen ligand (41), in good agreement with 18 U.S.C. Section 1734 solely to indicate this fact.

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agreement with the crystallographic data. The putative molybdenum ligand in the sequence of DmsA is Ser-176 on the basis of the sequence alignment. Molybdenum ligation by selenocysteine in \textit{E. coli} formate dehydrogenase H (FdHf) has been identified by using a combination of site-directed mutagenesis, isotope enrichment, and EPR (42). However, the similarity between the region encompassing the molybdenum ligand in FdHf and that in DmsA is significantly weaker than the similarity between DmsA and other \textit{S}- and \textit{N}-oxides, including \textit{Rh. sphaeroides} DMSR (43). In the sequence of the cofactor binding subunit of NarGHI, NarG, a Cys is found at the position occupied by a Ser in the \textit{N}- and \textit{S}-oxide reductases and the position occupied by a selenocysteine in FdHf (Fig. 1 (13), consistent with one of the sulfur in the ligation sphere being from this Cys residue. Overall, there appears to be good agreement between the crystallographic and spectroscopic data on the nature of the molybdenum ligation sphere in the bacterial molybdenoenzymes.

In this paper, we have used site-directed mutagenesis and EPR spectroscopy to examine the role of Ser-176 of DmsA in molybdenum ligation. This residue is mutated to either an Ala, which could not provide a ligand to the molybdenum, or Ser-176 codon were generated through oligonucleotide-directed mutagenesis of single-stranded pDMS223 DNA using the Sculptor kit and mutagenic primers, which substituted the Ser codon (TCC) for Ala (GCC), Cys (TGC), or His (CAC). Mutants were sequenced, subcloned into pDMS160 using EcoRI and EcoRV, and resequenced to confirm the mutation.

**Harvesting of Cells and Preparation of Membrane Fractions**—For membrane preparations, glycerol/fumarate-grown cells were harvested, washed, and resuspended in 50 mM MOPS, pH 7.0, 5 mM EDTA. Phenylenediamine and benzyl viologen (0.2 mM) was added to washed cells that were subjected to French pressure lysis and centrifugation for 1 h at 40,000 rpm in a Beckman T50.2 rotor (2). The membranes were washed with 100 mM MOPS, pH 7.0, 5 mM EDTA and resuspended in the same buffer. Membranes were stored at −70°C prior to use.

**Protein Determination**—Protein concentrations were estimated by a modification of the Lowry procedure (45) using a Bio-Rad bovine serum albumin protein standard.

**Enzyme Assays**—The ability of the mutant DmsABC enzymes to oxidize reduced benzyl viologen (BV⁺) in a Me₂SO- or TMAO-dependent manner was assayed as described previously (6). One unit of specific activity corresponds to 1 μmol of BV⁺ oxidized min⁻¹ mg⁻¹ protein.

**Quantitative Molybdenum and Molybdopterin**—The amount of molybdenum present in the membrane preparations containing overexpressed enzymes was determined by Alpha Laboratory Services (Edmonton, Canada) by using the Form A assay (46). 20 mg of membrane protein were acidified with HCl, 2 volumes of 2% KI, 1% I₂ were added, and the sample was boiled for 20 min. Samples were centrifuged, and the supernatant was analyzed by fluorescence spectroscopy (5).

**EPR Spectroscopy**—Redox titrations of washed membranes were carried out as described by Camaack and Weiner (6) using sodium dithionite and potassium ferricyanide to alter the redox potential. The mediators used in the redox titrations were quinhydrone, 2,6-dichlorophenolindophenol, toluylene blue, methylene blue, resolufin, indigo trisulfonate, indigo carmine, anthraquinone 2-sulfonic acid, benzyl viologen, and methyl viologen. Spectra were recorded using a Bruker ESP 300 EPR spectrometer equipped with an Oxford Instruments ESR-900 flowing helium cryostat. Instrument conditions and temperatures are described in the individual figure legends. Spin quantitations were determined by double integration of spectra obtained under nonsaturating conditions using a Cu-EDTA standard (47). For the molybdopterin quantitations an additional correction was made to account for not integrating the hyperfine lines due to molybdenum with a nuclear spin of 5/2 (48).

**RESULTS**

**Characterization of the Ser-176 Mutants**—\textit{E. coli} DSS301 is unable to grow anaerobically on glycerol-Me₂SO medium unless complemented with a plasmid containing the \textit{dmsABC} operon. None of the three mutant enzymes, expressed in DSS301, are able to support growth using Me₂SO as the terminal electron acceptor (Table I). HB101 contains a wild-type copy of the \textit{dmsABC} operon on the chromosome and grows on glycerol-Me₂SO, but this growth is completely inhibited when this strain is transformed with the Ser-176 mutant plasmids (data not shown). Plasmid-expressed enzyme competes for membrane assembly with the small amount of enzyme produced from the chromosomal operon, a phenomenon that has been noted in previous studies of DmsABC mutant enzymes (7, 49). The expression (Table I) and membrane content of the mutant enzymes are normal compared with the wild type. DmsABC oxidizes BV⁺ in a TMAO- or Me₂SO-dependent manner (Table I). This ability is reduced to a level much lower than...
The concentration of [Fe-S] was divided by 4 to estimate the concentration of enzyme.

| Enzyme activities of membranes containing amplified levels of wild-type and mutant DmsABC |
|-----------------------------------------------|
| Plasmid | Growth | Specific activities |            |
|        |        | BV⁺:TMAO | BV⁺:Me₂SO | TMAO/Me₂SO |
| pBR322  | NG     | 16 ± 1   | 1.5 ± 0.2 | 10.7       |
| pDMS160 | +      | 96 ± 10  | 10 ± 2   | 9.6        |
| pS176A  | NG     | 3.6 ± 0.8| 0.4 ± 0.1| 9          |
| pS176C  | NG     | 3.1 ± 0.5| 0.36 ± 0.07| 8.6      |
| pS176H  | NG     | 2.8 ± 0.8| 0.29 ± 0.03| 9.7      |

* Growth of plasmid containing DSS301 was determined from measurements of turbidity in glycerol/Me₂SO medium using a Klett-Summerson spectrophotometer. NG, no growth; +, growth.

Washed membranes were prepared from HB101 cells grown on glycerol/fumarate medium. Activities are expressed in units of μmol of BV⁺ oxidized min⁻¹ mg⁻¹ protein.

that of the background (HB101/pBR322) in the mutant enzymes, indicating that catalytically inactive plasmid-encoded enzyme is competing with active chromosomally-encoded enzyme. HB101 was chosen as the host for expression of DmsABC, rather than DSS301, due to the large amounts of fumarate reductase in DSS301 membranes that interferes with EPR analyses (7, 9).

All three mutant enzymes contain both molybdenum and molybdopterin (Table II); the amount of molybdenum in the enzyme varied among the different preparations. The wild-type enzyme and the S176A mutant have essentially one molybdenum atom per mol of enzyme, but the amount of molybdenum relative to enzyme is decreased in the S176C and S176H mutants. S176C has 0.78 mol of molybdenum per mol of enzyme, whereas S176H has 0.71 mol of molybdenum per mol of enzyme. The relative amount of fluorescence detected in the Form A assays of each of the enzyme preparations parallels that of the molybdenum concentration, with S176C and S176H having wild-type levels of molybdopterin, whereas S176C and S176H have reduced levels of molybdopterin. This agrees with previous studies of DmsABC that indicate molybdopterin is not inserted into DmsA in the absence of molybdenum (5).

**Molybdenum Ligation in DmsABC**

**TABLE I**

| Enzyme activities of membranes containing amplified levels of wild-type and mutant DmsABC |
|-----------------------------------------------|
| Enzyme | Signal intensities | Normalized fluorescence | Midpoint potentials, E₉₅₋₇₁ |
|        | amplitude | area | Em | | | |
|        | nMol mg⁻¹ protein |BV⁺:TMAO | BV⁺:Me₂SO | TMAO/Me₂SO | Mo(VI)/Mo(V) | Mo(V)/Mo(IV) |
| DmsABC | 0.40 | 0.32 | 0.39 | 0.39 | 15 | 175 |
| S176A  | 0.45 | 0.41 | 0.44 | 0.39 | — | 35 |
| S176C  | 0.41 | 0.16 | 0.32 | 0.34 | 27, 34 | —, 7, 33 |
| S176H  | 0.38 | — | 0.27 | 0.25 | — | — |

* [Fe-S] concentrations were estimated by double integration of spectra of dithionite-reduced membrane samples obtained at 12 K and 2 mW. The concentration of [Fe-S] was divided by 4 to estimate the concentration of enzyme.

* Molybdenum concentrations were estimated from spectra obtained at 95 K under the following nonsaturating conditions: DmsABC, −91 mV and 50 mV; S176A, 351 mV and 2 mW; S176C, 23 mV and 2 mW; S176H, 59 mV and 2 mW.

* The total amount of molybdenum in the membrane preparations was determined by inductively coupled plasma emission spectroscopy.

* Form A-pterin fluorescence at an emission wavelength of 455 nm with an excitation wavelength of 380 nm. Fluorescence intensities in arbitrary units are normalized to the molybdenum content of membranes containing wild-type DmsABC.

* — no value given.

* S176C midpoint potentials given are for the g = 1.992 peak and the g = 1.985 peak-trough.

**TABLE II**

**Spin quantitation and midpoint potentials of the EPR signals from HB101 expressing wild-type or mutant DmsABC**

**Redox Titrations of the Wild-type DmsABC Mo(V) Signal**—Redox titrations of DmsABC and the Ser-176 mutant enzymes were carried out to determine the midpoint potentials of the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples. A titration of the wild-type enzyme is shown in Fig. 3A. Of the three oxidation states through which the molybdenum cycles, only Mo(V) is paramagnetic so the titration curve shows the appearance and disappearance of this intermediate oxidation state. The DmsABC Mo(V) signal appears at approximately −250 mV, increases to a maximum at −91 mV, and then decreases until, at approximately 85 mV, it is no longer visible. The data from the titration can be fitted to a model of the Nernst equation with two consecutive midpoint potentials (E₉₅₋₇₁) for the reduction of Mo(VI)/Mo(V) and Mo(V)/Mo(IV). The E₉₅₋₇₁ of the Mo(VI)/Mo(V) couple is −15 mV, and the E₉₅₋₇₁ of the Mo(V)/Mo(IV) couple is −175 mV, with an average of −95 mV. The amount of Mo(V) estimated by double integration of the spectrum of the E₉₅₋₇₁ sample and the total amount of molybdenum present are shown in Table II. In DmsABC, approximately 90% of the total molybdenum was EPR visible at −91 mV.

**Redox Titrations of the Mo(V) Signals in the Ser-176 Mutants**—In redox titrations of S176A membranes, the Mo(V) signal appears at approximately −50 mV and increases to a maximum intensity above 125 mV (Fig. 3A). The appearance of the Mo(V) EPR signal of the S176A mutant can be fitted to a single midpoint potential of 35 mV. At least 90% of the molybdenum signal is detectable in each titration experiment.
denium is oxidized to Mo(V), which even at 200 μM ferricyanide (spectrum shown in Fig. 2) remained in the Mo(V) state.

Spectra from a redox titration of the S176C membranes are shown in Fig. 4. Analyses of redox titrations of the S176C mutant indicate that several Mo(V) species are present. The two largest features, a peak at g = 1.992 and a peak-trough at g = 1.985, behave independently, indicating that they arise from different species. The redox titration curves for these two features are shown in Fig. 3B and the midpoint potentials are in Table II. Integration of the Eh = -24 mV spectrum indicates that, at this potential, the spectrum dominated by the g = 1.992 species accounted for 40% of the total molybdenum in this enzyme. The Eh = 346 mV spectrum in Fig. 4 is not equivalent to the ferricyanide oxidized-spectrum in Fig. 2C, suggesting that the high Eh induced by ferricyanide may cause the appearance of an additional Mo(V) species not observed during potentiometric redox titrations.

The S176H membranes were titrated over a range of Eh from -435 to 350 mV, but a Mo(V) signal was not detected. Fluorescence analyses of molybdopterin content and analyses of molybdenum content (Table II) indicate that all of the mutant enzymes contain a similar amount of Mo-MGD. Thus, in the case of the S176H mutant, the paramagnetic Mo(V) redox state appears to be inaccessible.

**EPR Spectra of Dithionite-reduced Membranes—HB101 membrane preparations were reduced with dithionite and EPR spectra recorded at 12 K to examine the [4Fe-4S] clusters in the mutant enzymes (Fig. 5).** The background strain, HB101/pBR322, expresses very little DmsABC, and the spectrum shows primarily the features of fumarate reductase (8, 51). The spectrum of HB101/pDMS160 membranes is identical to that of DmsABC in membranes as previously characterized and has been interpreted as two pairs of interacting [4Fe-4S] clusters (Em,7 = -50, -120, -240, and -340 mV) (6–9). The spectrum contains peaks at g = 2.05, 2.03, 1.99, and 1.95 and two troughs located at g = 1.92 and g = 1.88. Fumarate reductase contributes to the peak at g = 2.03 and the peak-trough at g = 1.95 to g = 1.92. The reduced spectra of the S176C and S176H mutants are very similar to the wild-type spectrum. However, the line shape of the S176C spectrum (Fig. 5D) is altered; the peaks at g = 2.05 and g = 1.95 are increased in size, and the g = 1.94 shoulder is more distinct. The content of [Fe-S] clusters is not increased in this mutant, and only four phases are present in analyses of the g = 2.05 and g = 1.95 features from redox titrations of S176C membranes indicating that all four [4Fe-4S] clusters are present. The midpoint potential of the Em,7 = -240 mV [4Fe-4S] cluster appears to be shifted to a more positive potential in this mutant (>100mV more positive, data not shown). The extent of this change in Em,7 could not be accurately quantified because the Mo(V) interacts magnetically with the reduced [Fe-S] clusters (6), modifying their spectra, and complicating assignments of their Em,7 values. These observations indicate that there is a possible conformational link between the Em,7 = -240 mV [4Fe-4S] cluster of DmaB and the molybdenum binding region of DmaA.

**Fig. 2. Mo(V) EPR spectra of HB101 membranes.** a, HB101/pDMS160; b, HB101/pS176A; c, HB101/pS176C; and d, HB101/pS176H. Sample a, was poised at -91 mV during a redox titration. Samples b–d were incubated at 25 °C with 200 μM ferricyanide for 10 min before freezing in liquid nitrogen. Spectra were recorded under the following conditions: temperature, 95 K; microwave power, 20 mW; microwave frequency, 9.45 GHz; modulation amplitude, 3.02 Gpp at 100 KHz. Spectra were corrected for the amount of enzyme present as determined from spin quantitations (Table II).

**Fig. 3. Redox titration curves of the Mo(V) signals in HB101 membranes.** A, the change in signal amplitude is plotted as a function of redox potential for the g = 1.982 signals of HB101/pDMS160 (squares) and HB101/pS176A (diamonds) membranes. B, the change in signal amplitude of the g = 1.992 peak (circles) and the g = 1.985 peak-trough (triangles) of the HB101/pS176C membranes is plotted as a function of redox potential. Spectra were recorded under the conditions outlined in Fig. 2.
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**DISCUSSION**

DmsA is the catalytic subunit of *E. coli* Me$_2$SO reductase and contains a Mo-MGD cofactor. It belongs to a family of prokaryotic molybdoenzyme subunits that share sequence homology and probably ligate the same molybdopterin cofactor (1, 13). The determination of the crystal structure of *Rh. sphaeroides* DMSR (38) and a variety of biophysical studies have provided insights into the nature of the molybdenum ligation in this group of enzymes (41–43). Aided by sequence alignments (Fig. 1) and the crystal structure of DMSR (38), we have mutated a ligand of molybdenum in DmsA. Mutation of Ser-176 has a profound effect both on the EPR line shape and the redox properties of the molybdenum. In the case of the S176A mutant, the molybdenum is trapped in the Mo(V) state at high Eh. Substituting Cys at this position causes a heterogeneity in the molybdenum environment and also appears to affect at least one of the [Fe-S] clusters. Substituting His prevents the appearance of Mo(V) through the range of Eh studied. Overall, the effects of mutating Ser-176 are consistent with its proposed role in molybdenum ligation.

Mutating Ser-176 to Ala increases the gav and the g1–g3 of the Mo(V) EPR spectrum. Chicken liver sulfite oxidase exhibits low and high pH Mo(V) spectra (52, 53). The high pH Mo(V) species has a higher gav and g1−g3 than the low pH species, in a manner similar to the wild-type and S176A Mo(V) species studied herein. EXAFS has been used to correlate the line shape change between the low and high pH forms of sulfite oxidase species with a loss of a molybdenum ligand (53). It is likely, given these studies on sulfite oxidase, and taking into consideration the molybdenum ligation sphere of the *Rh. sphaeroides* DMSR revealed by x-ray crystallography, that the line shape change elicited by the S176A mutation in DmsA is the result of the loss of a molybdenum ligand, Ser-176.

In terms of interpreting the effects of the mutation on the Mo(V) EPR line shape, the situation with the S176C mutant is complicated by the multiple species detected. There is a large difference between the high Eh ferricyanide-oxidized species and the two major species observed at lower Eh values in potentiometric titrations. The difference between the two low Eh species and the high Eh species is reminiscent of the difference between the wild-type and the S176A mutant. It is therefore possible that the high Eh S176C species has fewer ligands than the low Eh species. An explanation for this stems from the conformation changes observed in the pterin dithiolene ligation of the molybdenum in *Rh. sphaeroides* DMSR. Of the two pterin dithiolenes, one (the Q-pterin) interacts less strongly than the other (the P-pterin) (38). It is possible that a subpopulation of the Cys of the S176C mutant is able to form a disulfide bridge with one of the Q-pterin dithiolene sulfurs, preventing direct ligation of the molybdenum, resulting in the high gav, high g1−g3 line shape. At low Eh, only the forms with a Cys ligand to the molybdenum would be observed, and these would be observed in potentiometric titrations. Comparison of Fig. 2C and Fig. 4 (346 mV spectrum) suggests that the putative disulfide bond may become reduced under anaerobic conditions, as the high Eh ferricyanide-oxidized species is not observed in reoxytations at high Eh.

Of the DmsA mutants, the S176H mutant is the most difficult to interpret, due to the absence of any EPR visible Mo(V) species detected in our studies. We had anticipated that possible nitrogen ligation of the molybdenum in this mutant would result in EPR spectra in which nitrogen hyperfine coupling would be observed. Unfortunately, we are unable to draw any conclusions concerning the molybdenum ligation sphere from

![Fig. 5. Spectra of the reduced membranes at 12 K.](image-url)
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this mutant; the only clear result is that the redox potentials of the molybdenum are shifted, so that a Mo(V) species is not observed in the $E_m$ range studied.

In the other bacterial molybdenzyme for which molybdenum ligand mutagenesis data are available, *E. coli* FdH:F, a Se-Cys has been shown to be a molybdenum ligand (42). Enrichment of the enzyme with $^{77}$Se (1) (1 = 1/2) causes hyperfine splitting of the Mo(V) EPR signal indicating a direct coordination of selenium to the molybdenum. Mutagenesis of FdH:F to replace the Se-Cys with Cys causes a major change in the line shape and the $g$ values of the Mo(V) signal: both the $g_{av}$ and the anisotropy ($g_1 - g_3$) are decreased. Changes in EPR parameters resulting from a change in the coordination of the molybdenum are also observed in the molybdenum hydroxylases when comparing the native and desulfo forms of the enzymes (54). The native enzymes have one o xo and one sulphydryl ligand to Mo(V), which becomes one o xo and one hydroxyl ligand in the inactive desulfo forms. Comparison of Mo(V) signals indicates that replacement of the sulphydryl ligand with a hydroxyl group decreases the $g_{av}$ and the $g_1 - g_3$ (54, 55). Conversely, replacing an oxygen ligand with a sulfur ligand should increase the $g_{av}$ and the $g_1 - g_3$. In potentiometric titrations of the S176C mutant (Fig. 4), the apparent $g_{av}$ is increased compared to the wild-type Mo(V) spectrum (Fig. 2A).

Comparisons of the electrochemical and EPR properties of the molybdenum of DmsA and DMSR reveal two important differences between the two enzymes. First, in DMSR, the $E_{m,7}$ of the Mo(V/IV) ($E_{m,7}$ = 200 mV) and Mo(V/IV) ($E_{m,7}$ = 141 mV) couples are more appropriate for Me$_3$SO ($E_{m,7}$ = 160 mV) reduction than is the case for DmsA (20). Second, DMSR has been shown to contain a coupled proton to the molybdenum that is observable through hyperfine splittings of the Mo(V) EPR spectrum (20). Such a proton has not been observed in DmsA (6).

In Rh. sphaeroides DMSR, the structure of the enzyme reveals a Tyr residue (Tyr-114) that provides a hydrogen bond to the o xo group of the Mo=O moiety of the oxidized enzyme. There is no equivalent Tyr in the sequence of DmsA (38), and the identity of potential amino acid residues providing a hydrogen bond to the o xo group is currently unknown. It is not clear if the Tyr of DMSR is implicated in the observation of a coupled proton in this enzyme. The differences in electrochemical and EPR properties between the two enzymes will probably be rationalized through site-directed mutagenesis studies of amino acid residues close to the molybdenum and the MGDs.

The $E_{m,7}$ of the Mo(V)/Mo(IV) couple in the S176A mutant is 35 mV, 210 mV more positive than that of the wild-type Mo(V)/Mo(IV) couple, $E_{m,7}$ = −175 mV. The enzyme is trapped in the Mo(V) state at $E_s$ values increasing to 450 mV. Thus, in the S176A mutant the molybdenum is unable to transfer two electrons to Me$_3$SO, and this is probably a major cause of this mutant’s inability to catalyze Me$_3$SO reduction.

Several different Mo(V) species were detected in redox titrations of the S176C mutant. The two largest signals come from species with $E_{m,7}$ values of 27 and 34 mV for the Mo(VI)/Mo(V) couples and $E_{m,7}$ values of −7 and −33 mV for the Mo(V)/Mo(IV) couples. The $E_{m,7}$ values of the individual species are closer together and shifted to a more positive potential than those of the wild-type enzyme but less than the S176A mutant.

Comparison of the molybdenum $E_{m,7}$ values of the S176C mutant indicates that the major species should be able to participate in Me$_3$SO reduction, so it is unclear why this mutant is catalytically inactive.

The Cys substitution of Ser-176 also alters the line shape of the reduced [4Fe-4S] clusters as well. The molybdenum in DmsA is known to interact magnetically with the $E_{m,7}$ = −120 mV [4Fe-4S] cluster of DmsB (6). The S176C mutation appears to affect the $E_{m,7}$ = −240 mV [4Fe-4S] cluster indicating that this cluster may be near to Ser-176 in DmsABC. It is not known how the molybdenum center in DmsA becomes reduced, but the electrons may come sequentially from one [4Fe-4S] cluster or possibly from two [4Fe-4S] clusters (perhaps the $E_{m,7}$ = −120 and −240 mV centers). The $E_{m,7}$ = −50 mV center has been implicated in interactions with a menaquinol binding site in DmsC (9). Little information is available about the $E_{m,7}$ = −340 mV center which may play a structural role or could be involved in mediating electron transfer. Further studies on the [Fe-S] clusters and the Mo-MGD will be aimed at understanding these subunit interactions and electron transfer between the subunits.

No Mo(V) signal was visible in redox titrations of S176H, although the mutant enzyme has molybdopterin and molybdenum. The midpoint potentials of the S176H molybdenum center may be shifted to a higher potential than that of ferricyanide, as it is unlikely that they would be significantly less than −435 mV. The lowest midpoint potential determined for a molybdenum couple is −470 mV at pH 7.7 for *Methanobacterium formicicum* formate dehydrogenase (22).

The $E_{m,7}$ values of the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) couples are dependent on the molybdenum environment. In the native form of the molybdenum hydroxylases (oxygen and sulfur coordination), one or both $E_{m}$ values are higher than the $E_{m}$ values of the desulfo enzymes (oxygen coordination) (55). In the DmsA family of molybdoenzymes the identity of the ligand, Se-Cys, Cys, or Ser, may influence the redox properties of the molybdenum. The molybdenum midpoint potentials of a Se-Cys containing enzyme have not been determined but must be low to be able to catalyze the oxidation of formate ($E_{m,7}$ = −425 mV for the formate/CO$_2$ couple). DmsABC ($E_{m,7}$ = −15 and −175 mV) and Me$_3$SO reductase from Rh. sphaeroides ($E_{m,7}$ = 144 and 200 mV (20)) have intermediate midpoint potentials and Ser coordination of the molybdenum. *E. coli* nitrate reductase (Cys residue) has $E_{m}$ values of 180 and 220 mV at pH 7.1 (23). The formate dehydrogenase from *M. formicicum* which has both a Cys and a sulphydryl ligand (54) has the lowest $E_{m}$ of all the molybdoenzymes characterized, $E_{m,7}$ = −330 and −470 mV at pH 7.7 (22), indicating that other elements in the molybdenum environment are important for determining the molybdenum $E_{m}$ values.

The $E_{m,7}$ values obtained from the titration of wild-type DmsABC in membranes are −15 mV for the Mo(VI)/Mo(IV) couple and −175 mV for the Mo(V)/Mo(IV) couple, with an average of −95 mV. The $E_{m,7}$ values determined in an earlier study of DmsABC containing membranes were −75 and −90 mV, with an average of −83 mV (6). In the previous study, the $E_{m,7}$ values, but not the Mo(V) signal itself, were highly dependent upon the preparation of DmsABC and the ratio of Mo(V) to enzyme was 0.33. DmsABC loses cofactor during purification, and the purified enzyme retains approximately 30% of its Mo-MGD (5, 28). The amount of Mo(V) detected in membranes used for the current study accounts for over 80% of the molybdenum that should be present if each enzyme molecule has one molybdenum (Table II). Care has been taken in this study to avoid repeated freeze-thaw cycles and unnecessary manipulations to prevent the loss of molybdenum. In contrast to the previous study, these membranes did not exhibit a partial Mo(V) signal at potentials greater than 80 mV (6).

Overall, the data presented herein show the consequences of changing the Mo-protein ligand of *E. coli* DmsABC. Loss of ligation by DmsA-Ser176 causes major changes in the redox and EPR properties of the enzyme and the molybdenum center.
The S176A mutant is unable to cycle through the Mo(V) ↔
Mo(VI) redox states. The S176C mutant causes significant
heterogeneity of the molybdenum center, and in the S176H
mutant the Mo(V) state is inaccessible. These studies provide
new insights into the role of molybdenum ligation in bis-
(MGD)-Mo-containing bacterial molybdoenzymes.

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