Spectroscopic and Kinetic Studies of Y114F and W116F Mutants of Me$_2$SO Reductase from Rhodobacter capsulatus*

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Mutants of the active site residues Trp-116 and Tyr-114 of the molybdenum-containing Me$_2$SO reductase from Rhodobacter capsulatus have been examined spectroscopically and kinetically. The Y114F mutant has an increased rate constant for oxygen atom transfer from Me$_2$SO to reduced enzyme, the result of lower stability of the $E_{red}$-Me$_2$SO complex. The absorption spectrum of this species (but not that of either oxidized or reduced enzyme) is significantly perturbed in the mutant relative to wild-type enzyme, consistent with Tyr-114 interacting with bound Me$_2$SO. The as-isolated W116F mutant is only five-coordinate, with one of the two equivalents of the pyranopterin cofactor found in the enzyme dissociated from the molybdenum and replaced by a second MoO$\equiv$O group. Reduction of the mutant with sodium dithionite and reoxidation with Me$_2$SO, however, regenerates the long-wavelength absorbance of functional enzyme, although the wavelength maximum is shifted to 670 nm from the 720 nm of wild-type enzyme. This "redox-cycled" mutant exhibits a Me$_2$SO reducing activity and over-all reaction mechanism similar to that of wild-type enzyme but rapidly reverts to the inactive five-coordinate form in the course of turnover.

Dimethyl sulfoxide reductase catalyzes the reduction of dimethyl sulfoxide (Me$_2$SO) to dimethyl sulfide (DMS)$^3$ and is a member of a large class of mononuclear molybdenum-containing enzymes (1–3) having an L$_2$MoO(X) core (where L represents a pyranopterin cofactor coordinated to the molybdenum via an enedithiolate side chain, and X represents a ligand in most cases provided by the polypeptide, a serinate in the case of Me$_2$SO reductase). Two forms of Me$_2$SO reductase exist, as exemplified by the enzymes from Escherichia coli (a heterotramer consisting of the DmsABC gene products) and Rhodobacter species (the monomeric DorA protein). Both are components of anaerobic respiratory chains and catalyze the reductive abstraction of oxygen from Me$_2$SO according to the following stoichiometry using electrons derived from the respiratory pool.

$$\text{Me}_2\text{S}=\text{O} + 2e^- + 2\text{H}^+ \rightarrow \text{Me}_2\text{S} + \text{H}_2\text{O}$$

(Eq. 1)

The Rhodobacter sphaeroides DorA enzyme is a soluble periplasmic protein of 85 kDa possessing the molybdenum cofactor as the sole redox-active center. Because of the distinctive absorption features of its molybdenum center, the DorA enzyme has become a paradigm for understanding the electronic structure of molybdenum centers of the L$_2$MoO(X) variety (4–12). In addition to steady-state kinetics under a variety of conditions, rapid reaction studies with both the Rhodobacter capsulatus (10) and R. sphaeroides enzyme (13) have also been reported. With the R. sphaeroides enzyme, the reaction of reduced enzyme with Me$_2$SO at low pH is biphasic. A fast [Me$_2$SO]-dependent phase ($k_{lim} \sim 1000$ s$^{-1}$) yields a spectrally distinct $E_{red}$-Me$_2$SO complex, with absorption maxima at 490 and 550 nm, followed by a slower oxygen abstraction step (35 s$^{-1}$) that yields DMS and $E_{ox}$ (13). The hyperbolic dependence of the rapid phase on [Me$_2$SO] gives a $K_{d,Me_2SO}$ of $\sim 155$ $\mu$M and indicates that the observed $E_{red}$-Me$_2$SO species is not the initial Michaelis complex but instead is the product of its breakdown. Oxidized enzyme also reacts with excess DMS to give this same $E_{red}$-Me$_2$SO species (5, 14), demonstrating the reversibility of the oxygen atom transfer reaction.

The crystal structure of Me$_2$SO reductase (14–18) suggests that two active site residues, Trp-116 and Tyr-114, are of particular interest. Trp-116 hydrogen-bonds to the labile MoO$\equiv$O group of oxidized enzyme and, thus, may be directly involved in catalysis. The role of Tyr-114 is less clear, although it also is close to the molybdenum center and has been suggested to hydrogen-bond to the oxygen atom of bound Me$_2$SO in the $E_{red}$Me$_2$SO complex (24, 25). With development of recombinant expression systems (in E. coli in the case of the R. sphaeroides enzyme (21, 22) or homologously in R. capsulatus (23)), both Tyr-114 (24, 25) and Trp-116 (26) have been targeted for mutation. The Y114F mutant turns over substantially more rapidly with both Me$_2$SO and trimethylamine-N-oxide (TMAO, an alternate oxidizing substrate) relative to the wild-type enzyme in steady-state assays, although this increase in rate is offset by a decrease in substrate affinity (24, 25). Steady-state analysis (25) has yielded a $k_{cat}$ of 81.4 s$^{-1}$ and $K_{m}$ of 185.5 $\mu$M for the Y114F mutant of the R. capsulatus enzyme, which...

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$^3$ The abbreviations used are: DMS, dimethyl sulfide; DCIP, dichlorophenolindophenol; Me$_2$SO, dimethyl sulfoxide; PES, phenazine ethosulfate; TMAO, trimethylamine-N-oxide; Bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; CHES, 2-(cyclohexylamino)ethanesulfonic acid; qdt, quinonoxaline-2,3-dihiolate.

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compare with values of 42.9 s⁻¹ and Kₘ of 9.7 μM for wild-type enzyme. It has thus been suggested that Tyr-114 stabilizes the substrate-bound intermediate E₇₅    reduced Me₂SO reductase (24, 25). Initial characterization of the W116F mutant from R. capsulatus showed that its molybdenum center is five-coordinate, with one of the pterin ligands of the LMoO₂ coordination sphere (that designated crystallographically as “Q”) replaced by a second Mo=O (26). The mutant enzyme exhibited ~10% of wild-type catalytic activity. The present work reports a detailed spectroscopic and rapid reaction kinetic study of wild-type R. capsulatus Me₂SO reductase and the Y114F and W116F mutants and provides new insights into the structural and functional roles of Tyr-114 and Trp-116 in the wild-type enzyme.

EXPERIMENTAL PROCEDURES

Protein Purification; Purification of Recombinant Me₂SO Reductase—R. capsulatus strain 37b4 carrying the recombinant dorA gene and its mutants, generated as described previously (23, 25), was grown phototrophically using malate as the carbon source and in the presence of 10% of wild-type catalytic enzyme (Fig. 1). Oxidized and reduced, and substrate-bound intermediate enzyme. It has thus been suggested that Tyr-114 stabilizes the reaction yields the relative contributions of each of the parent spectra. Further manipulations were performed using SigmaPlot Version 8.0. Because sodium dithionite (which absorbs strongly in the near-UV) was used as reductant, spectral analysis was restricted to wavelengths above 400 nm. Simulations of these reactions were performed using Applied Photophysics software. In some cases (e.g. with the Y114F mutant and Me₂SO as substrate) the generally similar component spectra for oxidized, Mo(V), and E₇₅    reduced Me₂SO enzyme forms required fitting of the experimental data by hand. All these experiments were performed in 50 mM KH₂PO₄, 0.6 mM EDTA, pH 6.0.

pH Dependence of DMS Oxidation—Steady-state assays of phenazine ethosulfate-dependent DMS:DCIP oxidoreductase activity were performed as described previously (10). The activity was measured with DMS (20 mM) and PES (0.2 mM) using DCIP (0.04 mM) as the electron acceptor; all assays were performed anaerobically. Activity was calculated from the loss of 600-nm absorbance using an ε₆₀₀ of 23.0 mm⁻¹ cm⁻¹ for DCIP corrected for background activity (measured at each pH in the absence of enzyme). Buffers used for the pH dependence were 50 mM KH₂PO₄, 0.6 mM EDTA, pH 6.0, 50 mM Bis-Tris, 0.6 mM EDTA, pH 7.0, 50 mM Tris-HCl, 0.6 mM EDTA (pH 7.5, 8.0, 8.5), 100 mM CHES, 0.6 mM EDTA, pH 9.0, and 100 mM borate, 0.6 mM EDTA (pH 9.5 and 10.0).

Electron Paramagnetic Resonance Spectroscopy—Samples for EPR characterization of R. capsulatus enzyme forms were prepared by mixing oxidized enzyme and made anaerobic in a glass tonometer with a concentrated dithionite solution (either with or without TMAO) followed by rapid freezing in a liquid nitrogen cooled acetone bath. EPR measurements were carried out using a Brüker ESP 300 spectrometer with the following instrument settings: modulation frequency of 100 kHz, modulation amplitude of 5 gauss, sweep width of 300 gauss (from 3250–3550 gauss), and 2 milliwatts of microwave power.

Resonance Raman—Samples for resonance Raman studies were prepared in 10 mM KH₂PO₄, pH 6.0; EDTA was removed, and a low buffer concentration was used to minimize the possibility of any interference due to phosphate or EDTA vibrational modes. Samples were concentrated to 1–3 mM by Microcon ultrafiltration at 5°C. Cryogenic samples (25–35 μl) were frozen by placing the protein solution in the sample well of a nickel-plated sample holder affixed to the cold finger of an APD Cryogenics Inc. liquid helium cryostat. Resonance Raman spectra were obtained and calibrated using previously described instrumentation and methods (26). The PEAKFIT program from Jandel Scientific was used to determine Raman band positions with a maximal error of ±2 cm⁻¹. Base-line correction was performed by polynomial fits using an in-house MATLAB Version 6.1.0.450 macro (The MathWorks, Inc.) running on a SGI Octane2 work station; corrected spectra were plotted using SigmaPlot Version 8.0.

RESULTS

UV-visible Spectroscopy of Me₂SO Reductase Mutants—Fig. 1 shows the UV-visible spectra of the (redox-cycled) oxidized, reduced, and E₇₅    reduced Me₂SO forms of wild-type, Y114F, and W116F Me₂SO reductase at pH 6.0. The spectra for wild-type enzyme (Fig. 1A) are well characterized (21–25). Oxidized enzyme has an absorption maximum at 720 nm (indicative of
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FIGURE 1. UV-visible spectra recorded for wild-type (panel A) and Y114F (panel B) Me$_2$SO reductase. Shown are spectra for oxidized (solid line), E$_{red}$Me$_2$SO complex (dashed line), and reduced (long dashed line) intermediates. UV-visible spectra recorded for W116F Me$_2$SO reductase (panel C) in the as-isolated (dashed line), redox-cycled/oxidized (solid line), and reduced (long dashed line) forms. Inset, spectra for redox-cycled (solid line) and redox-cycled after addition of 10 mM DMS (dotted line). Spectra were recorded in 50 mM KH$_2$PO$_4$, 0.6 mM EDTA, pH 6.0.

bis(enedithiolate) coordination and shoulders at $\sim$560, 470, and 360 nm; the reduced enzyme has absorption bands at 640 and 380 nm; the E$_{red}$Me$_2$SO complex has absorption maxima at 560 and 485 nm. For the Tyr-114 mutant (Fig. 1B), as seen previously, the spectrum of the oxidized enzyme is very similar to that seen for the wild-type enzyme (24, 25). Such differences as do exist include a modest bleaching in the near UV (best evidenced by loss of the band centered at 350 nm) and a slight red-shift of the long-wavelength absorbance from 720 nm in the wild-type enzyme to 730 nm. The spectrum of the reduced Y114F mutant is also similar to that of the wild-type enzyme but with some extinction loss observed in the 375-nm absorption maximum and a red-shift in the long-wavelength absorption from 640 to 660 nm. On the other hand, as previously observed by Ridge et al. (25), the spectrum of the E$_{red}$Me$_2$SO complex is significantly perturbed upon mutation of Tyr-114 to phenylalanine. Compared with the spectrum of the wild-type complex, which has strong absorbance maxima at 485 and 550 nm, the complex seen with the Y114F mutant has significantly weaker absorption bands at 470 and 565 nm. That the E$_{red}$Me$_2$SO spectrum is so perturbed upon mutation is consistent with Tyr-114 being involved in hydrogen-bonding to the oxygen of the complexed Me$_2$SO (24, 25).

The as-isolated W116F mutant lacks appreciable long-wavelength absorbance (Fig. 1C). On the basis of the similarity of the spectrum to that for the pentacoordinate “HEPES-modified” form of the wild-type enzyme, in which the Q pterin has dissociated from the molybdenum (20), the mutant enzyme is also thought also to be predominantly five-coordinate (26). Redox-cycling oxidized W116F mutant by reduction with MV$^-$ and reoxidation with Me$_2$SO results in a spectrum with the characteristic long-wavelength absorption of bis(enedithiolate) coordination, albeit blue-shifted to 680 nm relative to the 740 nm of wild-type enzyme (Fig. 1C). We conclude that the oxidized W116F mutant regains bis(enedithiolate) coordination upon redox-cycling. The spectrum of the reduced W116F mutant is also perturbed relative to that of the wild-type enzyme and resembles that seen for the E$_{red}$Me$_2$SO complex of wild-type enzyme (rather than the reduced form), with absorption maxima at 470 and 550 nm (as compared with 470 and 565 nm for wild-type enzyme). Surprisingly, the addition of DMS (to a concentration of 10 mM) to the oxidized W116F enzyme results in a uniform bleaching over the entire visible spectrum, with little if any indication of the characteristic spectrum of the E$_{red}$Me$_2$SO complex of wild-type enzyme formed under these conditions. The spectrum seen with the mutant in fact resembles that of the reduced “as-isolated” pentacoordinate form of the enzyme (20), suggesting that the Q pterin has dissociated upon treatment with DMS (Fig. 1C, inset).

Oxidative and Reductive Half-reaction Kinetics of Wild-type and Y114F Me$_2$SO Reductase—To better understand the cata lytic behavior of R. capsulatus Me$_2$SO reductase, the rapid reaction kinetics of reduced wild-type enzyme and mutants was examined. Our previous work was done with the wild-type R. sphaeroides enzyme, and the work here with the wild-type R. capsulatus enzyme was done to ensure proper comparison with the R. capsulatus mutants that were the primary focus. Measurements were performed at pH 6.0, 25 °C and are summarized in Table 1. In the reaction of reduced enzyme with Me$_2$SO, the E$_{red}$Me$_2$SO species formed in the 2–3-ms dead time of the stopped-flow apparatus with both the wild-type and Y114F mutant R. capsulatus enzyme (consistent with the limiting rate of reaction seen with the R. sphaeroides enzyme of $\sim$1000 s$^{-1}$; Ref. 13). With wild-type enzyme this intermediate
TABLE 1
Pre-steady-state kinetic data obtained for wild-type, Y114F, and W116F recombinant Me₂SO reductase

| Enzyme form | Oxidative half reaction | Reductive half-reaction |
|-------------|-------------------------|-------------------------|
|             | 5 °C | 25 °C | Mo(VI)/Mo(V) | Mo(V)/Mo(IV) | Mo(V)/Mo(V) |
| Wild type   | 13 ± 1 | 43 ± 1.7 | 4.9 ± 0.2 | 0.83 ± 0.06 |
| Y114F       | 28 ± 1 | 103 ± 4.3 | 1.2 ± 0.03 | 0.19 ± 0.02 |
| W116F       | 14 ± 1 | 61 ± 1.2 | 0.74 ± 0.05 | 0.41 ± 0.05 |

Broke down to oxidized enzyme with a rate constant of 43 s⁻¹, comparable to the 38 s⁻¹ seen with the R. sphaeroides enzyme and in excellent agreement with the previously reported steady-state kcat of 42.9 s⁻¹ (25). With the Y114F mutant, the ErredMe₂SO complex decayed to Eox with a rate 103 s⁻¹, approximately twice as fast as seen with wild-type enzyme and in satisfactory agreement with the reported steady-state kcat for the mutant of 81.4 s⁻¹ (25).

Reoxidation of reduced wild-type Me₂SO reductase by Me₂SO is known to be incomplete, with a significant amount of the ErredMe₂SO complex remaining at the end of the reaction (20). Fig. 2 shows the initial and final spectra observed during the reaction of reduced enzyme with Me₂SO for the wild-type enzyme (2A) and Y114F mutant (2B) along with the independently determined spectra for the oxidized enzyme forms. Fits of the final resting spectrum for wild-type enzyme using the known spectra for oxidized and ErredMe₂SO forms suggests that only ~60% of the enzyme becomes fully oxidized at the completion of the reaction, consistent with earlier work (20). On the other hand, the spectrum seen at the end of reaction with the Y114F mutant is virtually identical to that for the oxidized enzyme, implying that the ErredMe₂SO ⇌ Eox + DMS equilibrium lies further to the right for the mutant than is the case with the wild-type enzyme. This is consistent with the conclusion that Tyr-114 interacts with bound Me₂SO; its mutation to Phe both increases the rate of decay of the ErredMe₂SO intermediate and decreases its thermodynamic stability.

We next examined the reaction of oxidized enzyme with the non-physiological reductant sodium dithionite as a surrogate for the physiological DorC cytochrome. As expected on the basis of previous work with the R. sphaeroides enzyme (13), reduction of the recombinant wild-type R. capsulatus enzyme (Fig. 3A) was biphasic, with an absorption spectrum very similar to that of the “high-g split” Mo(V) intermediate, with wild-type R. sphaeroides Me₂SO reductase first appearing, then decaying to that of the fully reduced enzyme. As with the R. sphaeroides enzyme, decay of the Mo(V) species exhibited non-exponential behavior for reasons that are not understood. Nevertheless, at pH 6.0 and 5 °C, fits to the data (in the reaction of 100 μM wild-type enzyme with ~25 μM dithionite) yielded rate constants of 5 and 0.8 s⁻¹ for the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) transitions, respectively. The Mo(V) species also accumulated to a significant degree in the reaction of the Y114F mutant with dithionite, with rate constants for formation and decay of 1 and ~0.2 s⁻¹, respectively. The Y114F mutant, thus, reacts somewhat more sluggishly with dithionite than does wild-type enzyme.

To confirm that the intermediate seen with both wild-type and Y114F enzyme indeed represented the absorption spectrum of a Mo(V) species, freeze-quench EPR experiments were performed. The insets of Figs. 3, A and B, show the EPR spectra seen in the course of the reduction with dithionite, with sample being frozen within 5 s of the addition of dithionite. The observed signals are indistinguishable from the catalytically relevant high-g split Mo(V) signal described previously (9, 13) with wild-type enzyme, indicating that despite its proximity Tyr-114 does not interact to any significant degree with the molybdenum center in the Mo(V) state.
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Me₂SO reductase (13), and with the spectra of the several catalytically relevant species characterized above we have extended this approach to the mutant forms. In such experiments enzyme (at a sufficiently high concentration that its absorption spectrum can be accurately monitored) is mixed with excess concentrations of both Me₂SO (or TMAO) as oxidizing substrate and dithionite as reducing substrate. There is a rapid approach to the steady state which is subsequently maintained until the limiting substrate is exhausted. Spectra observed in the course of turnover may be treated as the weighted sum of the component spectra of all catalytically important enzyme species (e.g. $E_{ox}$, $E_{red}$ and the high-g split species) and deconvoluted to give quantitative time courses for each species (see “Experimental Procedures”).

With wild-type $R$. capsulatus enzyme recapitulated our earlier work with the wild-type $R$. sphaeroides enzyme but were necessary for proper comparison with the $R$. capsulatus mutants.

With wild-type $R$. capsulatus enzyme and TMAO as oxidizing substrate, the enzyme-monitored turnover analysis yielded comparable results to those seen with wild-type enzyme. Again, the spectrum seen in the steady state reflected a high degree of accumulation of the high-g split Mo(V) intermediate. With Me₂SO rather than TMAO as oxidizing substrate, the additional spectrum of the $E_{red}$-Me₂SO complex (obtained by mixing oxidized enzyme with 100 mM DMS) was needed in the spectral deconvolution. The time courses for each of the four catalytically relevant species (oxidized, reduced, high-g split, and $E_{red}$-Me₂SO) are shown in Fig. 4A. The results of the analysis were similar, albeit not identical, to those seen with the $R$. sphaeroides enzyme. In particular, the $E_{red}$-Me₂SO complex accumulates more rapidly and to a greater extent with the $R$. capsulatus enzyme than with the $R$. sphaeroides enzyme, and the high-g split Mo(V) species, thus, does not predominate to the same degree. Fits of the time course yielded an effective rate constant for oxygen atom transfer of 0.08 s⁻¹ in the steady-state, reflecting a very low level of turnover. This could be attributed to the large accumulation of the $E_{red}$-Me₂SO intermediate (~90% of the total enzyme concentration) as the product DMS accumulates and rebinds $E_{ox}$ to give $E_{red}$-Me₂SO to an increasing degree. This indicates that the $R$. capsulatus enzyme has an even higher affinity for DMS than does the $R$. sphaeroides enzyme and is even more susceptible to product inhibition.

With the Y114F mutant of the $R$. capsulatus enzyme and TMAO as oxidizing substrate, the enzyme-monitored turnover analysis yielded comparable results to those seen with wild-type enzyme. Again, the spectrum seen in the steady state reflected a high accumulation of the high-g split Mo(V) species (data not shown). On the other hand, with Me₂SO as oxidizing substrate, the additional spectrum of the $E_{red}$-Me₂SO complex (obtained by mixing oxidized enzyme with 100 mM DMS) was needed in the spectral deconvolution. The time courses for each of the four catalytically relevant species (oxidized, reduced, high-g split, and $E_{red}$-Me₂SO) are shown in Fig. 4A. The results of the analysis were similar, albeit not identical, to those seen with the $R$. sphaeroides enzyme. In particular, the $E_{red}$-Me₂SO complex accumulates more rapidly and to a greater extent with the $R$. capsulatus enzyme than with the $R$. sphaeroides enzyme, and the high-g split Mo(V) species, thus, does not predominate to the same degree. Fits of the time course yielded an effective rate constant for oxygen atom transfer of 0.08 s⁻¹ in the steady-state, reflecting a very low level of turnover. This could be attributed to the large accumulation of the $E_{red}$-Me₂SO intermediate (~90% of the total enzyme concentration) as the product DMS accumulates and rebinds $E_{ox}$ to give $E_{red}$-Me₂SO to an increasing degree. This indicates that the $R$. capsulatus enzyme has an even higher affinity for DMS than does the $R$. sphaeroides enzyme and is even more susceptible to product inhibition.

Enzyme-monitored Turnover with Wild-type and Y114F Me₂SO Reductase—We have recently demonstrated the utility of enzyme-monitored turnover studies with the $R$. sphaeroides enzyme (13), and with the spectra of the several catalytically relevant species characterized above we have extended this approach to the mutant forms. In such experiments enzyme (at a sufficiently high concentration that its absorption spectrum can be accurately monitored) is mixed with excess concentrations of both Me₂SO (or TMAO) as oxidizing substrate and dithionite as reducing substrate. There is a rapid approach to the steady state which is subsequently maintained until the limiting substrate is exhausted. Spectra observed in the course of turnover may be treated as the weighted sum of the component spectra of all catalytically important enzyme species (e.g. $E_{ox}$, $E_{red}$ and the high-g split species) and deconvoluted to give quantitative time courses for each species (see “Experimental Procedures”). Again, studies with the wild-type $R$. capsulatus enzyme recapitulated our earlier work with the wild-type $R$. sphaeroides enzyme but were necessary for proper comparison with the $R$. capsulatus mutants.

With wild-type $R$. capsulatus enzyme and TMAO as oxidizing substrate, the enzyme-monitored turnover analysis yielded comparable results to those seen with wild-type enzyme. Again, the spectrum seen in the steady state reflected a high degree of accumulation of the high-g split Mo(V) intermediate. With Me₂SO rather than TMAO as oxidizing substrate, the additional spectrum of the $E_{red}$-Me₂SO complex (obtained by mixing oxidized enzyme with 100 mM DMS) was needed in the spectral deconvolution. The time courses for each of the four catalytically relevant species (oxidized, reduced, high-g split, and $E_{red}$-Me₂SO) are shown in Fig. 4A. The results of the analysis were similar, albeit not identical, to those seen with the $R$. sphaeroides enzyme. In particular, the $E_{red}$-Me₂SO complex accumulates more rapidly and to a greater extent with the $R$. capsulatus enzyme than with the $R$. sphaeroides enzyme, and the high-g split Mo(V) species, thus, does not predominate to the same degree. Fits of the time course yielded an effective rate constant for oxygen atom transfer of 0.08 s⁻¹ in the steady-state, reflecting a very low level of turnover. This could be attributed to the large accumulation of the $E_{red}$-Me₂SO intermediate (~90% of the total enzyme concentration) as the product DMS accumulates and rebinds $E_{ox}$ to give $E_{red}$-Me₂SO to an increasing degree. This indicates that the $R$. capsulatus enzyme has an even higher affinity for DMS than does the $R$. sphaeroides enzyme and is even more susceptible to product inhibition.

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With the Y114F mutant of the $R$. capsulatus enzyme and TMAO as oxidizing substrate, the enzyme-monitored turnover analysis yielded comparable results to those seen with wild-type enzyme. Again, the spectrum seen in the steady state reflected a high degree of accumulation of the high-g split Mo(V) species (data not shown). On the other hand, with Me₂SO as oxidizing substrate, the additional spectrum of the $E_{red}$-Me₂SO complex (obtained by mixing oxidized enzyme with 100 mM DMS) was needed in the spectral deconvolution. The time courses for each of the four catalytically relevant species (oxidized, reduced, high-g split, and $E_{red}$-Me₂SO) are shown in Fig. 4A. The results of the analysis were similar, albeit not identical, to those seen with the $R$. sphaeroides enzyme. In particular, the $E_{red}$-Me₂SO complex accumulates more rapidly and to a greater extent with the $R$. capsulatus enzyme than with the $R$. sphaeroides enzyme, and the high-g split Mo(V) species, thus, does not predominate to the same degree. Fits of the time course yielded an effective rate constant for oxygen atom transfer of 0.08 s⁻¹ in the steady-state, reflecting a very low level of turnover. This could be attributed to the large accumulation of the $E_{red}$-Me₂SO intermediate (~90% of the total enzyme concentration) as the product DMS accumulates and rebinds $E_{ox}$ to give $E_{red}$-Me₂SO to an increasing degree. This indicates that the $R$. capsulatus enzyme has an even higher affinity for DMS than does the $R$. sphaeroides enzyme and is even more susceptible to product inhibition.
Spectroscopic and Kinetic Studies of Me$_2$SO Reductase Mutants

The Kinetic Behavior of W116F Me$_2$SO Reductase—Appreciating that the W116F mutant regains bis(enedithiolate) coordination on redox-cycling, the kinetic behavior of the mutant was next examined. Despite modest differences in its absorption spectrum (and presumably active-site coordination) (Fig. 1C), the reduced W116F mutant reacts very rapidly with Me$_2$SO to give the $E_{red}^{-}$Me$_2$SO intermediate, whose spectrum is comparable with that seen with wild-type enzyme. As with wild-type enzyme, this species subsequently decays rapidly to the oxidized form with the W116F mutant ($k_{cat} = 61$ s$^{-1}$; Table 1) considerably faster than the $7$ s$^{-1}$ seen for $k_{cat}$ in the steady-state assay with the mutant (25). The redox-cycled W116F mutant is, thus, fully catalytically competent. We attribute the relatively slow steady-state rate by Ridge and coworkers (25) to Q pterin dissociation (with concomitant loss of activity) in the course of repeated turnovers. As with the Y114F mutant, the end point spectrum seen in the reaction of the reduced W116F mutant with Me$_2$SO indicates no residual $E_{red}^{-}$Me$_2$SO complex formation (Fig. 2C). Again, the $E_{red}^{-}$Me$_2$SO \(\rightleftharpoons E_{ox}^{-}$ + DMS equilibrium lies further to the right for the W116F mutant than is seen with wild-type enzyme.

Although the reaction of reduced W116F Me$_2$SO reductase with Me$_2$SO is straightforward, the reductive half-reaction and enzyme-monitored turnover experiments are more complex. With the W116F mutant, significantly less Mo(V) intermediate accumulates in the course of the reaction with dithionite. As followed at 474 nm, an initial absorbance decrease is followed by an increase with rate constants of 0.74 and 0.41 s$^{-1}$ (Table 1). Simulation of the reaction using these rate constants gives 15, 45, and 40% of the enzyme as oxidized, Mo(V), and reduced forms, respectively, at maximum accumulation of the Mo(V) species (Fig. 2C). The absorption spectrum obtained for the Mo(V) species resembles that observed during reductive titrations of the non-functional pentacoordinate “as-isolated” enzyme (Fig. 1C), suggesting that the O pterin has dissociated from the molybdenum center in the course of reaction with dithionite.

Enzyme-monitored turnover experiments with the W116F mutant using either TMAO or Me$_2$SO as substrate are also complex, and in the case of the TMAO reaction, it is not possible to fit the observed spectra using only the established parent spectra (oxidized, reduced, Mo(V), and as-isolated). Given the difficulties in obtaining unique solutions using a larger number of parent spectra (including, e.g. that for the inactive five-coordinate species in both oxidized and reduced states), these data were not analyzed in greater detail. Furthermore, although the procedure used to obtain the high-$g$ split EPR signal with the other enzyme forms (turnover with TMAO and sodium dithionite) did yield a strong EPR signal (Fig. 3C, inset), this signal is fundamentally different from that observed for any other form of Me$_2$SO reductase. It is in fact similar to the low pH signal of Arabidopsis thaliana sulfite oxidase, with $g_{1,2,3}$ = 2.0070, 1.9760, 1.9654 (26, 29). Sulfite oxidase has an LMoO$_2$ center with single pyranopterin (as well as a cysteinyl ligand contributed by the protein), suggesting that the Q pterin has dissociated in the signal-giving species for the W116F mutant.

DMS Reduction of W116F Me$_2$SO Reductase—The propensity of even wild-type Me$_2$SO reductase to form catalytically inert forms has led to the suggestion (28) that a reverse assay involving the PES-dependent oxidation of DMS (rather than the conventional forward assay monitoring Me$_2$SO reduction) is a better indicator of the catalytic competence of the enzyme. In the presence of the mediator dyes PES (or phenazine methosulfate) and the oxidant DCIP, oxidation of DMS to Me$_2$SO can be monitored by the loss of absorbance of DCIP at 600 nm as the dye becomes reduced. Previous studies by Ridge et al. (27) failed to observe any reverse activity using as-isolated W116F Me$_2$SO reductase, consistent with the hypothesis that the absence of even wild-type Me$_2$SO reductase, consistent with the hypothesis that the
isolated mutant enzyme has a non-functional five-coordinate molybdenum center. As indicated above, however, redox-cycling the W116F mutant restores the functional bis(enedithiolate) coordination. We have, therefore, examined the pH dependence of activity in the reverse DMS:DCIP/PES assay. Fig. 5 shows that although the activity observed was small, the characteristic bell-shaped pH profile is clearly observed and is consistent with DMS:DCIP/PES activity observed for both wild-type and Y114F recombinant enzymes (25). The pH optimum for wild-type enzyme is pH 8.3, with two pKₐ values of 7.5 and 9.1 (25), whereas maximal activity was seen at pH 9.2 with corresponding pKₐ values of 8.8 and 9.6 (25) with the Y114F mutant. The large errors observed for the reverse assay with the W116F mutant were the result of both low activity as well as the fact that the activity observed was only about twice that of the base-line activity measured in the absence of enzyme. Although the activity observed for the W116F mutant was ~100-fold lower than that of either the wild-type or Y114F mutant, the pH optimum could reasonably be assigned to pH 8.8 yielding pKₐ values of 8.0 and 9.3. We attribute such activity as is observed to enzyme that is (transiently) bis(enedithiolate) coordinated in the steady state. The relatively low activity observed reflects the low level of this functional form of the enzyme. Nevertheless, that the same pH profile is seen with wild-type and both Y114F and W116F mutants indicates that neither Tyr-114 nor Trp-116 is responsible for the ionizations giving rise to the pH dependence of the wild-type enzyme.

**Resonance Raman of Wild-type and Mutant Me₂SO Reductases**—As a final probe of the molybdenum center in Me₂SO reductase, we have examined the wild-type enzyme and mutants by resonance Raman spectroscopy. In general, we find that the resonance Raman spectra of wild-type enzyme for the three principal catalytic intermediates, oxidized, reduced, and E_red,Me₂SO, are in very good agreement with the earlier work of Garton et al. using the *R. sphaeroides* enzyme (5). Key vibrational modes are given in Table 2. Fig. 6 shows the resonance Raman spectrum of the oxidized W116F and Y114F mutants compared with that for the recombinant wild-type enzyme (in all cases, redox-cycled to ensure bis(enedithiolate) coordination) using an excitation wavelength of 647.1 nm. Vibrational modes in the Mo—S stretching region (330–400 cm⁻¹) for the W116F mutant in general downshift slightly (no more than 5 cm⁻¹) as compared with the wild type (indicating slightly longer Mo—S bond distances in this mutant), but the overall form of the spectrum is not significantly changed. The Mo—S stretching region for the Y114F mutant is identical to that of the wild-type enzyme (with an error of ±2 cm⁻¹). For wild-type enzyme, the Mo=O stretching frequency is 871 cm⁻¹ with maximal enhancement with 568.2 nm excitation. The Mo=O stretching mode frequency for the Y114F mutant is also 870 cm⁻¹ with very low intensity at 647.1-nm excitation. For the W116F mutant, the Mo=O stretching frequency is 870 cm⁻¹, and the intensity of this mode relative to the neighboring mode at 856 cm⁻¹ is essentially unchanged with excitation wavelengths ranging from 488 to 647.1 nm. Of greater significance is the “sharpening” of the high energy C—C stretch at 1569 cm⁻¹ accompanied by an ~20-cm⁻¹ shift to lower energy in the W116F mutant. This indicates a weaker C—C bond and greater π-delocalization within the dithiolene unit, reflecting a more symmetrical disposition of the two enedithiolate units in the molybdenum coordination sphere for W116F relative to the wild-type enzyme.

**TABLE 2**

A comparison of resonance Raman vibrational mode center frequencies for *R. sphaeroides* (*R. sph*) Me₂SO reductase with *R. capsulatus* (*R. caps*) wild-type, Y114F, and W116F mutant Me₂SO reductase in the oxidized, dithionite-reduced, and DMS-reduced forms

All center frequencies are in cm⁻¹. ox, oxidized; red, reduced.

| Vibrational mode | R. sph ox | R. caps ox | Y114F ox | W116F ox | R. sph red | R. caps red | Y114F red | W116F red | R. sph E_red,Me₂SO | R. caps E_red,Me₂SO | Y114F E_red,Me₂SO |
|-----------------|----------|-----------|----------|----------|------------|------------|----------|----------|-----------------|-----------------|-----------------|
| Mo=O            | 864      | 871       | 870      | 870      | 513        | 513        | 517      | 513      | 516             | 516             | 513             |
| Mo=O (ser)      | 533      | 545       | 547      | 545      | 513        | 513        | 517      | 513      | 516             | 516             | 513             |
| Mo=O (ser)      | 336      | 333       | 335      | 328      | 346        | 346        | 346      | 346      | 348             | 348             | 354             |
| Mo=O (ser)      | 342      | 350       | 350      | 345      | 364        | 365        | 365      | 365      | 364             | 364             | 367             |
| Mo=O (ser)      | 370      | 370       | 370      | 368      | 385        | 384        | 384      | 384      | 384             | 384             | 383             |
| Mo=O (ser)      | 377      | 379       | 378      | 378      | 402        | 401        | 401      | 401      | 399             | 404             | 403             |
| C=O            | 856      | 855       | 854      | 856      | 858        | 858        | 853      | 854      | 858             | 855             | 860             |
| C=O            | 1004     | 992       | 999      | 997      | 1005       | 1002       | 1002     | 1000     | 1005            | 1003            | 1003            |
| C=O            | 1126     | 1123      | 1124     | 1128     | 1118       | 1129       | 1129     | 1128     | 1118            | 1116            | 1112            |
| C=O            | 1158     | 1154      | 1155     | 1158     | 1159       | 1155       | 1155     | 1158     | 1118            | 1116            | 1112            |
| C=O            | 1023     | 1026      | 1026     | 1024     | 1017       | 1013       | 1013     | 1010     | 1017            | 1014            | 1011            |
| C=O            | 1047     | 1046      | 1045     | 1047     | 1044       | 1045       | 1045     | 1045     | 1047            | 1045            | 1045            |
| C=O            | 1527     | 1526      | 1533     | 1526     | 1527       | 1524       | 1524     | 1525     | 1527            | 1525            | 1523            |
| C=O            | 1578     | 1587      | 1582     | 1569     | 1571       | 1565       | 1564     | 1572     | 1573            | 1573            | 1568            |
| Mo=O (Me₂SO)   | 497      | 495       |          |          | 862        | 863        |          |          |                 |                 |                 |
| S=O (Me₂SO)    |          |          |          |          |            |            |          |          |                 |                 |                 |
The resonance Raman spectrum of the Y114F mutant is nearly identical to that of wild-type enzyme above 1000 cm\(^{-1}\) with only a slight (5 cm\(^{-1}\)) downshift of the highest energy C=S stretch. Maintenance of the general Mo=S and C=S vibrational profile of the oxidized wild-type enzyme in the W116F and Y114F mutants clearly indicates retention of the full bis(enedithiolate) character of the molybdenum center in the (redox-cycled) mutants, consistent with the results discussed above.

The absorption spectrum of the dithionite-reduced W116F mutant resembles that of the wild-type \(E_{\text{red}}\)Me\(_2\)SO complex rather than free reduced enzyme, as reflected in absorption bands at 470 and 560 nm. Fig. 7 shows the resonance Raman spectra for the dithionite-reduced W116F as compared with the wild-type enzyme. Despite the difference in absorption spectra, it is evident that the dithionite-reduced W116F Me\(_2\)SO reductase is nearly vibrationally equivalent to the reduced wild-type enzyme. The only significant difference is in the high energy C=S stretch which shifts to slightly lower energy (\(-10\) cm\(^{-1}\)) in the W116F mutant.

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The resonance Raman spectra of the \(E_{\text{red}}\)Me\(_2\)SO complex with wild-type enzyme and Y114F mutant are shown in Fig. 8. The spectrum of wild-type enzyme exhibits modes at 495 and 863 cm\(^{-1}\) that are only seen when Me\(_2\)SO is present. These modes have been assigned to the Mo=O stretch and the S=O stretch of bound Me\(_2\)SO, respectively, based on the isotopic
shifts of these two modes upon $^{18}$O substitution (5). The similar Mo—S stretching regions of the wild-type reduced and $E_{\text{red}}$Me$_2$SO spectra indicate that the molybdenum center is reduced in the latter species. With the Y114F mutant resonance Raman spectrum, no Me$_2$SO modes at ~495 and 863 cm$^{-1}$ were observed (Fig. 8), indicating a significant weakening of the sulfur-oxygen bond. That the complex had indeed formed was reflected in the obvious color change to deep pink on the addition of DMS to the oxidized enzyme, an indicator of DMS to the oxidized enzyme, an indicator of

DISCUSSION

Here we report a comprehensive rapid kinetic study of wild-type R. capsulatus Me$_2$SO reductase as well as Y114F and W116F mutants, in conjunction with a detailed spectroscopic analysis of the several catalytically relevant forms. As expected, the kinetic and spectroscopic properties of the wild-type R. capsulatus enzyme are very similar to those of the closely related enzyme from R. sphaeroides, although it is more prone to product inhibition by DMS. Both Y114F and W116F mutants destabilize the $E_{\text{red}}$Me$_2$SO intermediate seen in the catalytic sequence and largely relieve the enzyme from such inhibition. The Y114F mutant reacts somewhat more rapidly with both Me$_2$SO and TMAO than does wild-type enzyme (but at the expense of reduced substrate specificity); it reacts with dithionate somewhat more slowly than does wild type but forms the same high-g split Mo(V) intermediate. In enzyme-monitored turnover experiments with the Y114F mutant, this Mo(V) species accumulates essentially quantitatively during turnover with TMAO. That the EPR spectrum is unchanged on mutation indicates that Tyr-114 does not interact significantly with the Mo(V) form of the molybdenum center in the wild-type enzyme. With the W116F mutant, the observed EPR signal is similar to that seen with A. thaliana sulfite oxidase at low pH (26, 29), most likely reflecting dissociation of the Q pterin from the molybdenum in the course of the reaction.

The W116F mutant is isolated in a non-functional five-coordinate state but is converted to the functional six-coordinate bis(enedithiolate) form by reduction and reoxidation with Me$_2$SO (so-called redox cycling). It is this form of the enzyme that likely accounts for the steady-state activity of the mutant reported previously (25), and indeed the reconstituted form of the W116F, once reduced, reacts somewhat more rapidly with Me$_2$SO than does the wild-type enzyme. In the context of earlier controversies regarding molybdenum coordination in the functional form of the enzyme, our results underscore that it is the bis(enedithiolate) form that is active, even in the W116F mutant.

The absorption spectra for the several catalytically relevant species (including oxidized, high-g split, $E_{\text{red}}$Me$_2$SO, and reduced forms) for wild-type enzyme and the Y114F and W116F mutants have also been determined. Mutation of Tyr-114 to Phe results in little change in the absorption spectrum of oxidized or reduced enzyme. The spectrum of the $E_{\text{red}}$Me$_2$SO complex of the Y114F mutant is significantly perturbed as compared with wild-type enzyme, consistent with the proposed hydrogen bond to bound Me$_2$SO in the $E_{\text{red}}$Me$_2$SO complex and the observed kinetic and thermodynamic destabilization of the intermediate. Mutation of Trp-116 to Phe, on the other hand, causes a blue shift of the long-wavelength absorption of (redox-cycled) oxidized mutant to 680 nm (from 720 nm for wild-type enzyme). Interestingly, the reduced W116F mutant resembles the $E_{\text{red}}$Me$_2$SO species of wild-type enzyme, a point discussed further below. The $E_{\text{red}}$Me$_2$SO species is destabilized to an even greater degree in the W116F mutant, to the point that the addition of DMS to the oxidized mutant (the most convenient way to form this species with wild-type or Y114F enzyme, albeit only incompletely in the latter case) does not yield the $E_{\text{red}}$Me$_2$SO species but, rather, the inactive, five-coordinate form of the mutant. The intermediate is observed transiently, however, in the reaction of the reduced W116F mutant with Me$_2$SO.

The spectrum of the reduced W116F mutant has absorption maxima at 470 and 550 nm and is reminiscent of that of the

FIGURE 8. Resonance Raman spectra for DMS-reduced recombinant wild-type (WT) and Y114F Me$_2$SO reductase. Spectra were recorded in 10 mM KH$_2$PO$_4$, pH 6.0, with excitation at 568.2 nm for wild type and 514.5 nm for Y114F.
in the case of the enzyme, but it is possible that ambient thermal energy may be sufficient to disrupt binding of solvent to the wild-type reduced center at room temperature. The implication is that Trp-116 serves to prevent formal binding of water, which may represent a first step in the process of Q pterin dissociation.

In any case, the data presented here suggest that turnover of the W116F mutant with Me$_2$SO involves several steps, as shown in Scheme 1. The pentacoordinate as-isolated species is first reduced to the solvent-bound bis(enedithiolate) Mo(V) species, passing through a Mo(V) species in which one of the sulfurs of the Q pterin enedithiolates has possibly re-coordinated to the metal (this species also appears during one-electron reduction of the bisdithiolene-oxidized enzyme). Water coordinates weakly to the reduced enzyme. The addition of Me$_2$SO to the reduced mutant displaces this water, leading to an oxidative half-reaction closely resembling that of the wild-type enzyme; that is, rapid formation of the $E_{\text{red}}$ Me$_2$SO species (via a preceding Michaelis complex) followed by oxygen atom abstraction to give the bis(enedithiolate)-coordinated oxidized enzyme. Unlike the wild-type enzyme, however, formation of the $E_{\text{red}}$ Me$_2$SO complex by rebinding of dissociated DMS to the oxidized mutant enzyme does not occur (consistent with the low activity observed for the non-physiological reverse assay). In fact, the addition of DMS to oxidized enzyme results in formation of the pentacoordinate as-isolated enzyme. A predisposition for Q pterin dissociation is likely the basis for the relatively low steady-state catalytic activity previously observed (26) for the W116F mutant (as distinct from the larger rate constants for single turnover as determined here). Our results indicate that W116F functions catalytically with full coordination to both pyranopterin cofactors, but that mutation of Trp-116 to Phe results in a greater propensity for the Q pterin to dissociate in the course of turnover.

Resonance Raman analysis of the Y114F and W116F mutants has also been undertaken. Importantly, once redox-cycled, our results indicate that the molybdenum center of the latter mutant possesses full bis(enedithiolate) coordination as seen in the wild-type enzyme, although Mo—S bond lengths are somewhat longer than in the wild-type enzyme (as evidenced by downshifting of a majority of the distinctive Mo—S stretching modes ($<400$ cm$^{-1}$)). There is also less double bond character and greater $\pi$-delocalization within the dithiolene moiety of the pyranopterin cofactor. The modest structural changes giving
rise to these vibrational differences appear to have relatively little effect on catalysis as the reaction of reduced enzyme with Me$_2$SO appears to be largely unaffected by the mutation.

The present work demonstrates the role of Tyr-114 in stabilizing the $E_{\text{red}}$Me$_2$SO complex encountered in the course of catalysis by hydrogen-bonding to the oxygen of bound substrate. Although not essential for enzyme turnover, it plays a role in substrate binding, as reflected in the effect of its mutation on $K_m$ as well as on the $E_{\text{ox}}$:DMS = $E_{\text{red}}$Me$_2$SO equilibrium in the active site. This may be of major significance in the context of the use of Me$_2$SO as an electron acceptor by bacteria in the environment since Me$_2$SO concentrations are often in the submicromolar range (3). Trp-116, on the other hand, appears to be important less from a strictly catalytic standpoint than in helping to maintain the structural integrity of the molybdenum active center. By inhibiting the binding of water to the reduced form of the enzyme, displacement of the Q$_{\text{red}}$ group is retarded.

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