Reactions of Dimethylsulfoxide Reductase from *Rhodobacter capsulatus* with Dimethyl Sulfide and with Dimethyl Sulfoxide: Complexities Revealed by Conventional and Stopped-Flow Spectrophotometry†

Benjamin Adams,‡ Andrew T. Smith,*,‡ Susan Bailey,§ Alastair G. McEwan,|| and Robert C. Bray⊥

School of Biological Sciences, University of Sussex, Brighton BN1 9QG, U.K., CLRC Daresbury Laboratory, Warrington WA4 4AD, U.K., Department of Microbiology, University of Queensland, Brisbane, Queensland 4072, Australia, and School of Chemistry, Physics and Environmental Science, University of Sussex, Brighton BN1 9QJ, U.K.

Received January 27, 1999; Revised Manuscript Received April 16, 1999

ABSTRACT: Improved assays for the molybdenum enzyme dimethylsulfoxide reductase (DMSOR) with dimethyl sulfoxide (DMSO) and with dimethyl sulfide (DMS) as substrates are described. Maximum activity was observed at pH 6.5 and below and at 8.3, respectively. Rapid-scan stopped-flow spectrophotometry has been used to investigate the reduction of the enzyme by DMS to a species previously characterized by its UV−visible spectrum [McAlpine, A. S., McEwan, A. G., and Bailey, S. (1998) *J. Mol. Biol. 275*, 613−623], and its subsequent reoxidation by DMSO. Both these two-electron reactions were faster than enzyme turnover under steady-state conditions, indicating that one-electron reactions with artificial dyes were rate-limiting. Second-order rate constants for the two-electron reduction and reoxidation reactions at pH 5.5 were $(1.9 \pm 0.1) \times 10^5$ and $(4.3 \pm 0.3) \times 10^2$ M^{-1} s^{-1}, respectively, while at pH 8.0, the catalytic step was rate-limiting (62 s^{-1}). Kinetically, for the two-electron reactions, the enzyme is more effective in DMS oxidation than in DMSO reduction. Reduction of DMSOR by DMS was incomplete below ∼1 mM DMS but complete at higher concentrations, implying that the enzyme's redox potential is slightly higher than that of the DMS−DMSO couple. In contrast, reoxidation of the DMS-reduced state by DMSO was always incomplete, regardless of the DMSO concentration. Evidence for the existence of a spectroscopically indistinguishable reduced state, which could not be reoxidized by DMSO, was obtained. Brief reaction (less than ∼15 min) of DMS with DMSOR was fully reversible on removal of the DMS. However, in the presence of excess DMS, a further slow reaction occurred aerobically, but not anaerobically, to yield a stable enzyme form having a λ_{max} at 660 mn. This state ($DMSOR_{mod}$) retained full activity in steady-state assays with DMSO, but was inactive toward DMS. It could however be reconverted to the original resting state by reduction with methyl viologen radical and reoxidation with DMSO. We suggest that in this enzyme form two of the dithiolene ligands of the molybdenum have dissociated and formed a disulfide. The implications of this new species are discussed in relation both to conflicting published information for DMSOR from X-ray crystallography and to previous spectroscopic data for its reduced forms.

Dimethylsulfoxide reductase (DMSOR)[1] contains molybdenum and the molybdopterin cofactor (*1*). Such enzymes (*2*−*5*) catalyze a variety of redox reactions that are essential for the cycling of nitrogen and sulfur compounds in mammals, plants, and bacteria. Recently determined crystal structures (*6*−*14*) represent a dramatic advance on earlier work with these enzymes (*15*−*17*). Most are complex enzymes depending on Moco [molybdopterin and its dinucleotide variants (*1, 18*)] and other redox centers, including iron−sulfur, heme, or flavin. The common component of all Moco forms is molybdopterin (MPT), a pterin with a four-carbon side chain with a phosphate group in the 4′-position and bearing a 1′,2′-ene-dithiolate (dithiolene) group, in which the dithiolene S atoms can act as Mo ligands. A variety of active site structures are based on the MPT nucleus. In the enzymes of higher organisms, unmodified MTP is utilized, while in microorganisms, the form most often observed is molybdopterin guanine dinucleotide (MGD). Crystal structures have shown that the pterin occurs as a tricyclic ring system (from condensation of the 3′-OH with the pterin 7-position), and that there may be either one or two MTP molecules ligated to a single Mo. In some of the enzymes, an amino acid residue also ligates the metal.

† This work was supported in part by a grant from the Wellcome Trust.

* To whom correspondence should be addressed.

‡ School of Biological Sciences, University of Sussex.

§ CLRC Daresbury Laboratory.

|| University of Queensland.

⊥ School of Chemistry, Physics and Environmental Science, University of Sussex.

[1] Abbreviations: DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide; DMSOR, dimethylsulfoxide reductase; $DMSOR_{mod}$, modified form of dimethylsulfoxide reductase formed on extended aerobic exposure to DMS; $DMSOR_{regen}$, enzyme regenerated from $DMSOR_{mod}$ by treatment with $MV^{\bullet}$ and DMSO and aerobic gel filtration, to restore the resting-state spectrum and enzyme activity; MPT, molybdopterin; MGD, molybdopterin guanine dinucleotide; PES, phenazine ethosulfate; PMS, phenazine methosulfate; DCPIP, 2,6-dichlorophenolindophenol; BV_{ox}, benzyl viologen; $BV^{\bullet}$, benzyl viologen radical; MV_{ox}, methyl viologen; $MV^{\bullet}$, methyl viologen radical.

10.1021/bi9902034 CCC: $18.00
Published on Web 06/06/1999

Study of the reactions of model molybdenum compounds (*19*) led to the proposal that these enzymes function via transfer of oxygen atoms in the form of oxo ligands of molybdenum. For two enzymes, oxygen transfer has indeed been well-established (*20*, *21*). However, we note (*22*) that, to date, for no molybdenum enzyme has a dioxo−monooxo catalytic cycle of the type envisaged by Holm (*19*) been rigorously established. Furthermore, in the case of the xanthine oxidase family of molybdopterin enzymes, it is now clear (*7*, *23*) that the transferred oxygen atom, though no doubt a ligand of the metal, is not an oxo group.

The DMSOR family (*2*) includes (*17*) respiratory nitrate reductases as well as enzymes acting on DMSO and closely related amine *N*-oxide reductases. DMSOR from the photosynthetic bacteria *Rhodobacter capsulatus* (*24*) and *Rhodobacter sphaeroides* (*25*), containing no heme, flavin, or iron−sulfur cofactors, is unusual among molybdenum enzymes in having this metal as its sole redox center. These two proteins (having sequences that are 77% identical) and recently trimethylamine *N*-oxide reductase from *Shewanella massilia* (*26*; sequence 45% identical to that of the *R. capsulatus* enzyme) have proved to be particularly amenable to study. The DMSOR of *Escherichia coli* (*27*−*29*; sequence 29% identical to the *R. capsulatus* enzyme) is also homologous, but contains an iron−sulfur center in addition to the molybdenum. All these enzymes have dual specificity for amine *N*-oxides and for DMSO, though only for the *E. coli* enzyme has this specificity been investigated in detail (*29*). DMSOR from *R. capsulatus* or *R. sphaeroides* enables DMSO to function as a periplasmic terminal electron acceptor for excess reducing power during anaerobic photosynthetic growth of these organisms, catalyzing a reaction of considerable environmental interest that leads to the release of DMS (*8*):

$$\mathrm{DMSO} + 2\mathrm{e}^- + 2\mathrm{H}^+ \leftrightarrow \mathrm{DMS} + \mathrm{H_2O}$$

Several X-ray crystallographic studies have been reported on these enzymes (*8*, *9*, *11*, *13*, *14*), complementing a variety of spectroscopic studies (*30*−*35*). All the structures contain two MGD molecules and a serine ligated to the molybdenum (S147 in the *R. capsulatus* enzyme). The combined results show that the active site of DMSO reductases can assume a number of different conformations, indicating that each of the Mo(IV), -(V), and -(VI) oxidation states can exist in more than one structural state. These appear to differ in the number of oxo ligands to the Mo and the number of sulfur atoms from the two cofactor molecules that, in addition to the serine ligand, are coordinated to the metal.

This apparent variety of structural conformations of the molybdenum center of DMSOR makes attempts to deduce the precise enzymatic mechanism difficult. Though as already noted, there is direct evidence that an oxygen atom is transferred in the catalytic reaction (*21*), the nature of the transferred oxygen (oxo, OH^-, or H_2O) has not been determined. Studies by resonance Raman spectroscopy [Garton et al. (*35*)] appear to favor a monooxo−desoxo catalytic cycle. Nevertheless, the authors concede (*35*) that they cannot exclude an alternative dioxo−monooxo cycle. Furthermore, this work lacks any fast kinetic component. Indeed, little conventional enzymological work has been reported for DMSOR, with almost no pre-steady-state kinetic studies (but see refs *32* and *36*) and with detailed kinetic work limited to the more complex *E. coli* enzyme. With molybdenum as its sole redox center, DMSOR is by far the simplest MPT enzyme, and though this has facilitated much of the spectroscopic work, surprisingly, the potential of UV−visible spectroscopy has so far been exploited little, with no kinetic work reported. This potential was greatly increased by the recent finding [Baugh et al. (*34*) and McAlpine et al. (*13*)] that the enzyme may be reduced by treatment with DMS to a spectroscopically and crystallographically distinct form. We present data on the reduction of DMSOR from *R. capsulatus* by DMS and on its subsequent reoxidation by DMSO, using both conventional and stopped-flow UV−visible spectroscopy. Though these reactions are faster than enzyme turnover, the work reveals unexpected complexities in both. Preliminary details of part of the work have been published (*37*).

MATERIALS AND METHODS

Enzyme Preparation. To produce maximum levels of DMSOR, *R. capsulatus* strain H123 was grown photosynthetically, under anaerobic conditions in the presence of 50 mM DMSO, using propionate as a carbon source (*24*). DMSOR was purified essentially as described by Bennett et al. (*32*). Briefly, spheroplasts were made by treatment with lysozyme. The periplasmic fraction was subjected to ammonium sulfate fractionation (DMSOR precipitating between 60 and 80% saturation), followed by hydrophobic interaction chromatography on phenyl Sepharose and finally by ion exchange on Q-Sepharose. The resultant enzyme had an A_{280}/A_{720} ratio of 81 ± 3, and an A_{625}/A_{720} ratio of 0.64 ± 0.02, and was pure according to polyacrylamide gel electrophoresis in the presence of SDS. Very minor spectral variations between enzyme batches were largely ascribed to light scattering. Enzyme concentrations were calculated from an ϵ_{720} of 2.00 mM^{-1} cm^{-1} (*32*).

Enzyme Assays. Unless otherwise stated, these were performed at 25 °C in 50 mM Tris-Cl^- and 1 mM EDTA (pH 8.0). DMSOR was assayed in the forward reaction (DMSO reduction) with $MV^\bullet$ or $BV^\bullet$ as electron donor (*25*, *38*). The procedure of Jones and Garland (*39*) was used[2] with no gas space over the solution, anaerobiosis being achieved without the use of nitrogen. The convenience of this assay was found to be much improved by the addition of catalase (4 μM heme[3]), which reduced from 15−20 min to a few seconds the time required for A_{600} to stabilize, as the sample was made anaerobic with $Na_2S_2O_4$. Slow stabilization is due to initially formed H_2O_2 reacting only slowly (*40*) with $MV^\bullet$. Catalase likewise improved the precision of the assay, by removing very rapidly the H_2O_2 generated by reduction of the small amount of dissolved oxygen usually introduced along with the substrate or enzyme in starting the assay. In conventional procedures, slow reaction of this H_2O_2 with $MV^\bullet$ will be superimposed on enzymatic $MV^\bullet$ consumption,

[2] Jones and Garland (*39*) specified the use of a stopper made of Teflon in closing the spectrophotometer cell. We found a nylon stopper preferable, because of the lower solubility of oxygen in this plastic.

[3] The use of catalase preparations containing alkylbenzyl dimethylammonium chloride added as a stabilizer was avoided, since this appeared to cause significant inhibition of the assay.

leading to overestimation of the rate. Standard assay conditions were 5 mM DMSO and 0.3 mM MV_{ox} or BV_{ox}. The reaction was followed at 600 nm to exhaustion of the reductant, and activity was calculated from the maximum rate of decrease of A_{600}, with $\Delta\epsilon_{600}$ values of 13.6 mM^{-1} cm^{-1} for $MV^{\bullet}$ (*41*) and 10.6 mM^{-1} cm^{-1} for $BV^{\bullet}$ (*42*). If desired, replicate measurements could be taken on the same assay mixture by further additions of $Na_2S_2O_4$.

Activity in the reverse direction (DMS oxidation) was assayed (*43*) with DMS (20 mM) and PES (0.2 mM) with DCPIP (0.04 mM) as electron acceptors. The reaction was followed aerobically at 600 nm. The activity was calculated from the initial rate, using a $\Delta\epsilon_{600}$ of 21.0 mM^{-1} cm^{-1} for DCPIP.

Ultraviolet−Visible Spectra. These were recorded using a Perkin-Elmer Lambda 16 spectrometer, and the data were analyzed by using the manufacturer's software and SigmaPlot (SPSS Science Software). Kinetic studies were carried out using an SX.18 MV stopped-flow apparatus equipped with a sequential mixing capability and a diode array rapid-scan system (Applied Photophysics). Data were analyzed using the manufacturer's software (Pro-K) and SigmaPlot.

DMS Solutions and Anaerobic Experiments. DMS (98%, Aldrich) was used as a 2 M solution in cold EtOH, diluted to give the nominal DMS concentrations indicated. The fresh solution in EtOH was sometimes a little turbid, presumably because of minor impurities in the DMS. Because of its volatility, actual concentrations of DMS are likely, depending on the precise experimental conditions, to be somewhat lower than the indicated nominal ones. Where there was evidence for serious losses of DMS, this is indicated in the text. Anaerobic addition of DMS to DMSOR was carried out in a glovebox operating at ~10 ppm O_2. The sample was subsequently removed in a tightly sealed spectrophotometer cell.

RESULTS

Forward Enzyme Assay (DMSO Reduction): Evidence for Enzyme Inhibition by Excess Viologen Radical. In the forward reaction, DMSOR is conventionally assayed (*38*) with $BV^{\bullet}$ or $MV^{\bullet}$ as the electron donor. Typical progress curves realized in this assay are illustrated in Figure 1. The rate of the enzyme-catalyzed reaction increases progressively as the viologen radical is consumed and until just before it becomes exhausted. The contrast between the reaction of $MV^{\bullet}$ at pH 8.0 and that of $BV^{\bullet}$ at pH 5.0 is illustrated (Figure 1a,b). Using $BV^{\bullet}$ at pH 8.0 resulted in a progress curve that was intermediate between the two shown. Thus, this acceleration is more marked for $BV^{\bullet}$ than for $MV^{\bullet}$, and at low pH than at high pH. By taking the first derivative (rate of change in absorbance) and plotting this against radical concentration (using $\Delta\epsilon_{600}$ values for $MV^{\bullet}$ and $BV^{\bullet}$ given in Materials and Methods), we obtained plots of reaction rate against radical concentration, as shown. The form of such curves is that typical for enzyme inhibition by excess substrate (in this case the radical), with the rate, v, governed by the equation

$$v = V_{max}/[1 + (K_s/S) + (S/K_i)]$$

This is shown by agreement of the data with the calculated

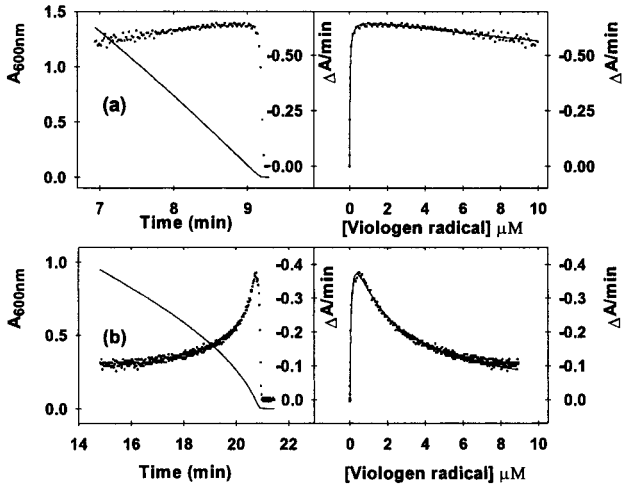


FIGURE 1: Analysis of progress curves for the forward enzyme assay (DMSO reduction): evidence for enzyme inhibition by excess viologen radical. (a) was obtained with $MV^{\bullet}$ at pH 8.0 and (b) with $BV^{\bullet}$ at pH 5.0. Absorption at 600 nm due to the viologen radical decreases progressively with time in the presence of the enzyme and DMSO (left-hand graphs; —). The corresponding rate measurements (ΔA per minute, calculated at 1.2 s intervals) are shown as individual data points (left-hand graphs; ●). The right-hand graphs show the same data, transformed into plots of ΔA per minute vs the viologen radical concentration calculated from A_{600}; individual data points are shown (●). The theoretical curves (—, right-hand graph) were calculated as described in the text, with K_s and K_i values in graphs a and b of 24 nM and 55 μM, and 80 nM and 1.8 μM, respectively. In a further experiment (data not shown) with $BV^{\bullet}$ at pH 8.0, values for K_s and K_i of 17 nM and 6.0 μM were obtained. Different enzyme concentrations were used in experiments whose results are depicted in graphs a and b; the initial MV_{ox} and BV_{ox} concentrations were 0.3 mM, and the initial DMSO concentration was 5 mM (for details of the assay procedure, see Materials and Methods).

curves shown in Figure 1. Values of the Michaelis constant (K_s) and the inhibition constant (K_i) derived from such curve fitting in a number of experiments are summarized in Table 1. For routine measurement of the enzyme activity in the standard assay, instead of calculating V_{max} by using the curve-fitting procedure, we used the maximum observed rate attained just prior to the substrate being exhausted. Data obtained in this way are summarized in Table 1. Particularly with the $MV^{\bullet}$ assay, results were highly reproducible. The accuracy of specific activity measurements was limited by the precision (about 5%) with which A_{720} measurements of the enzyme concentration could be taken for the dilute samples that were used.

The mechanism of the observed inhibition by excess viologen radical is uncertain, as is considered in the Discussion. Note that an alternative explanation would involve the accelerating rates being due to equilibrium between the viologen and dithionite redox couples. At low pH values and in the presence of sulfite, full reduction of the former is not necessarily expected [the redox potentials of the viologens are pH-independent; that of dithionite is both pH- and concentration- dependent (*41*)]. Thus, until the maximum rate was attained, free dithionite would be consumed along with the viologen radical, leading to diminished rates of absorption decrease (*28*). Such a mechanism is consistent with the observation that increasing the concentration of MV_{ox} (e.g., from 0.25 to 3.0 mM at pH 8.0) leads to the reaction continuing at very high initial

Table 1: Catalytic Properties of DMSOR from Steady-State Kinetic Experiments

enzyme	assay	catalytic center activity (s^{-1})[a]	substrate	K_s(app) (μM)[a]	inhibitor	K_i(app) (μM)[a]
DMSOR						
	MV•:DMSO	27 ± 1[b]	MV•	~0.02[c]	MV•	~50[c]
	BV•:DMSO	17 ± 1	BV•	~0.02[c]	BV•	~5[c]
	DMS:PES/DCPIP	8 ± 1	DMS	1000	DMSO	3[d]
$DMSOR_{mod}$[e]						
	MV•:DMSO	27 ± 1				
	DMS:PES/DCPIP	0.2 ± 0.1				
$DMSOR_{regen}$[f]						
	MV•:DMSO	23 ± 1				
	DMS:PES/DCPIP	6 ± 1				

[a] All data refer to 50 mM Tris buffer containing 1 mM EDTA at pH 8.0 and 25 °C, and generally to the standard assay conditions (see Materials and Methods). Catalytic center activities are expressed per two electrons and are based on an ϵ_{720} of 2.00 mM^{-1} cm^{-1}, except for $DMSOR_{mod}$ for which they are based on an ϵ_{660} of 2.49 mM^{-1} cm^{-1}. [b] Average value for the four different batches of enzyme used in this work which were prepared in three different laboratories. [c] Value deduced from analysis of the assay progress curves in a number of experiments, including those whose results are depicted in Figure 1. [d] Assuming competitive inhibition and based on 50% inhibition observed in the standard assay with 60 μM DMSO. [e] $DMSOR_{mod}$ is a modified form of DMSOR formed on extended aerobic exposure to DMS (see the legend of Figure 8). [f] $DMSOR_{regen}$ is $DMSOR_{mod}$ after regeneration by treatment, as in the assay with MV• (A_{600} ~ 1) for 2 min, followed by anaerobic addition of DMSO (5 mM) and aerobic gel filtration.

absorbances, instead of plateauing at an A of ~2, as at the lower MV_{ox} concentration (data not shown). On the other hand, it does not explain the observed differences between BV• and MV•. The redox potential of BV• (−360 mV) is higher than that of MV• [−440 mV (*42*)], so less marked rate acceleration would be predicted for the former than for the latter, which is contrary to the observed results (Figure 1).

Backward Enzyme Assay (DMS Oxidation). Little use has been made by previous workers of the backward reaction for the assay of DMSOR. We used the assay of Hanlon et al. (*43*, *44*) with PES and DCPIP as electron acceptors. Results, under the standard conditions with 50 mM Tris-Cl^- and 1 mM EDTA at pH 8.0 and 25 °C, are summarized in Table 1, with additional data. The value of K_s for DMS (1 mM) is relatively high, but DMSO was found to be a potent inhibitor of this assay, with an apparent K_i of ~3 μM. We found that PES could be replaced by PMS (activity increased by about 20%) and DCPIP by equine cytochrome *c*. However, the phenazine dye (PES or PMS) is essential for the reaction. Thus, negligible activity was found with DCPIP, with cytochrome *c*, with ferricyanide alone, or with DCPIP and either *p*-benzoquinone or *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine. We also attempted to measure, with an oxygen electrode, the ability of DMSOR to oxidize DMS (50 mM) using molecular oxygen, in air-saturated 50 mM Tris-Cl^- and 1 mM EDTA at pH 8.0 and 22 °C. Oxygen was consumed at a significant rate (3.1 ± 0.4 μM min^{-1}) in the absence of enzyme. We were unable to observe any increase in this rate on addition even of 11.5 μM enzyme. This enables us to set an upper limit for the catalytic center activity in this assay of 0.03 min^{-1}.

Effect of pH on the Activity of DMSOR. We have used both assays to determine the pH profiles of DMSOR activity (Figure 2). Whereas in the DMS:PES/DCPIP assay there is a sharp pH optimum at pH 8.3, for the BV•:DMSO assay, activity appears to be controlled by a single pK_a of ~8.8, giving an activity plateau in the low-pH region extending down as low as pH 5.0. Thus, activity at pH 5.5 is rather higher than that at pH 8.0, a result contrasting sharply with data in the literature (*25*; see also ref *11*).

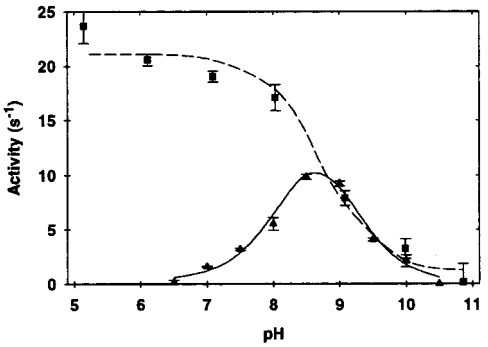


FIGURE 2: pH dependence of DMSOR activity in the forward and backward assays. DMSOR was assayed using both the forward BV•:DMSO assay (■) and the backward DMS:PES/DCPIP assay (▲). Activities are expressed as catalytic center activities per two electrons (cf. Table 1). To vary the pH, the standard buffer, 50 mM Tris (pH 8.0), was replaced with acetate (pH 5.0), MES (pH 6.0), MES (pH 6.5), MOPS (pH 7.0), MOPS (pH 7.5), BICINE (pH 8.0), BICINE (pH 8.5), CHES (pH 9.0), CHES (pH 9.5), CAPS (pH 10.0), CAPS (pH 10.5), or CAPS (pH 11.0), all at a concentration of 50 mM. EDTA at a concentration of 1 mM was included in all buffers. Assays were performed in quadruplicate, and error bars show standard deviations. The data were fitted assuming two pK_a values for the reverse assay and one pK_a value for the forward assay. The fitted curves correspond, for the DMS:PES/DCPIP data, to a lower pK_a of 8.30 ± 0.23 and a higher pK_a of 9.01 ± 0.25, and for the BV•:DMSO data to a single pK_a of 8.75 ± 0.20.

Binding of DMSO to DMSOR. We have found a clear change in the visible absorption spectrum of DMSOR in the presence of 150 mM DMSO (Figure 3). The peak at 723 nm in resting DMSOR is shifted to 711 nm, while other features are also somewhat changed. This is direct evidence for the formation of a complex between DMSOR and DMSO. DMSOR was titrated with DMSO, the position of the long-wavelength maximum being used as a measure of the extent of the binding of DMSO to DMSOR. The use of other measurement procedures was precluded by the small magnitudes of the absorbance changes. A fit of these data suggests that the dissociation constant for DMSO binding to DMSOR is 17 ± 4 mM. The spectral change was reversed (Figure 3) by gel filtration to remove the DMSO, the product retaining virtually full activity (catalytic center activity of 25 s^{-1}) in the standard forward assay (cf. Table 1).

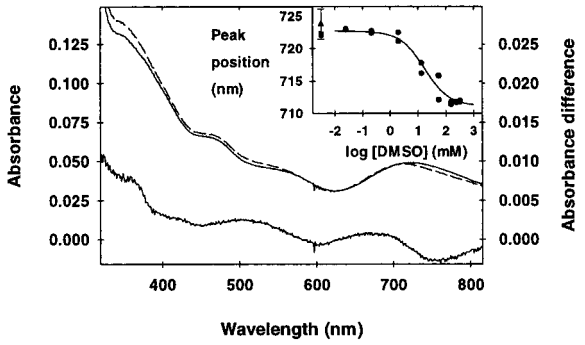


FIGURE 3: Effect of DMSO on the spectrum of DMSOR and evidence for the formation of a complex. The upper solid line of the main graph shows the spectrum of the enzyme alone, the dashed line that in the presence of DMSO (150 mM), and lower solid line the corresponding difference spectrum (right-hand axis). The experiment was carried out in 50 mM Tris-Cl^- buffer (pH 8.0) containing 1 mM EDTA, and the spectra were scaled to correct for dilution by the DMSO. The inset graph shows the position of the long-wavelength peak in the same experiment, as a function of the concentration of DMSO, following successive additions of this. The following symbols are used: (■) enzyme alone, (●) enzyme in the presence of DMSO, (▲) (with error bar) enzyme gel-filtered to remove DMSO after the experiment. The position of the long-wavelength spectral maximum was estimated from smoothed spectra using the software from the spectrophotometer manufacturer. The curve was calculated for a dissociation constant K_s of 17 ± 4 mM, with the peak shifting from 723 ± 1 to 711 ± 1 nm.

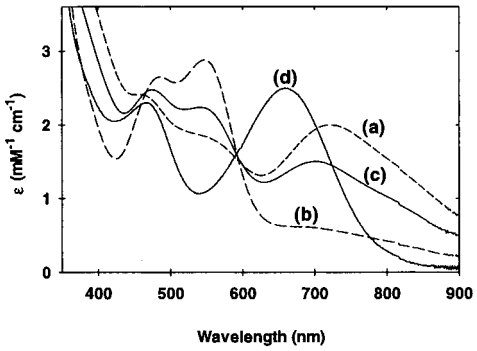


FIGURE 4: Absorption spectra of resting-state, reduced, DMSO-reoxidized, and DMS-modified DMSOR. The following spectra are shown: (a; - - -) resting air-oxidized DMSOR (as prepared), (b; - - -) DMSOR treated with 9 mM DMS for 2 min at 25 °C, (c; —) DMSOR treated with 10 mM DMS for 5 min and then with 80 mM DMSO for 5 min at 25 °C, and (d; —) DMSOR treated with 100 mM DMS for 22 h and then gel filtered into buffer ($DMSOR_{mod}$). All experiments were carried our aerobically in 50 mM Tris-Cl^- and 1 mM EDTA (pH 8.0). Absorbances are expressed as molar absorption coefficients (mM^{-1} cm^{-1}) based on an ϵ_{720} of 2.00 for the resting-state enzyme.

Reduction of DMSOR by DMS and Its Reoxidation by DMSO. (1) Preliminary Experiments Using Slow Manual Mixing. Typical data confirming the spectral change observed by McAlpine et al. (*13*) that occurs on adding (*34*) an excess of DMS to DMSOR are illustrated in Figure 4 (curves a and b). As discussed below, we studied this reaction under a variety of conditions. Under those described in the legend of Figure 4, complete reduction of the enzyme was attained, as indicated by the apparently complete disappearance of the 720 nm peak. With a DMS concentrations of less than ~1 mM, the reaction was incomplete. The reversibility of the reaction was shown by two sets of experiments (data not shown). At least for brief treatment (less than ~15 min at 25 °C) of the enzyme with DMS at pH 8.0, the resting-state spectrum (as in Figure 4, curve a) could be regained by simple removal of the DMS by gel filtration of the sample into the same buffer. The reoxidized DMSOR so obtained had a catalytic center activity in the DMS:PES/DCPIP assay that was unchanged within experimental error from that of the starting material (cf. Table 1). In a later experiment at the same pH in Bicine buffer, enzyme treated with sufficient DMS to give full reduction, and then simply incubated at 25 °C in an open spectrophotometer cell, was found after 50 min to have regained its original resting spectrum. The possibility that a weak DMS:O_2 oxidoreductase activity of the enzyme (see above) might have been responsible for this effect was considered. However, as the final spectrum was that of the resting enzyme and not that of the DMSO complex, the most probable explanation is that the DMS had simply evaporated from the solution. DMS boils at 38 °C, and according to Wood (*45*), its aqueous solutions have a particularly high vapor pressure (see Materials and Methods in relation to DMS concentrations).

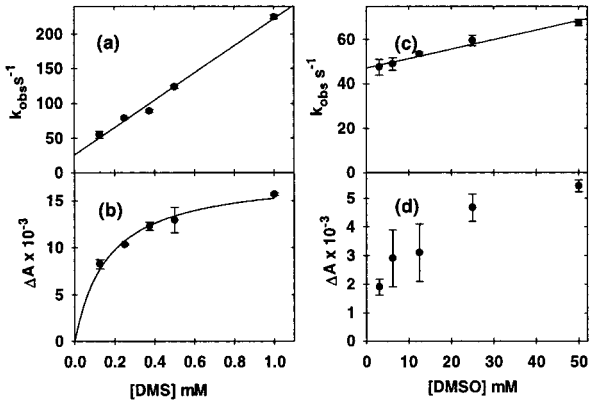


FIGURE 5: Stopped-flow kinetic data at pH 5.5 and 25 °C for the reduction of DMSOR by DMS and for its subsequent reoxidation by DMSO. For graphs a and b, DMSOR in 25 mM MES at pH 5.5 containing 1 mM EDTA was mixed with varying concentrations of DMS and the reaction was followed at 714 nm. Graph a shows the rate constant obtained, k_{obs}, plotted against the final DMS concentration, while graph b shows the total amplitude of the change, similarly plotted. Graphs c and d correspond to similar plots for the enzyme, first reduced with 0.5 mM DMS for a few minutes, and then mixed with varying concentrations DMSO. Theoretical curves through the data points were calculated as described in the text, and parameters derived from them are summarized in Table 2. The final enzyme concentration was 10.5 μM in all experiments.

Addition of a large excess of DMSO to DMS-reduced DMSOR was found to lead, as illustrated in Figure 4 (curve c), to reoxidation of the enzyme, though this was always incomplete (see below).

(2) Pre-Steady-State Kinetic Studies. We report stopped-flow studies on the reduction of DMSOR by DMS and on its subsequent reoxidation with DMSO. Experiments were carried out at pH 8.0 and 5.5, and both single-wavelength (714 nm) and rapid-scan diode array measurements were performed. Initial experiments (*37*) showed that the DMS-reduced species is formed rapidly on mixing the resting-state enzyme with DMS. At pH 8.0, the reaction with 10 mM DMS proceeds very rapidly and to completion, with an apparent first-order rate constant of >500 s^{-1} at 10 °C (data not shown). At pH 5.5 and 25 °C, this reduction was much slower (Figure 5a), with a linear dependence of the apparent first-order rate constant, k_{obs}, on the DMS concentration. This

Table 2: Catalytic Properties of DMSOR from Pre-Steady-State Kinetic Experiments Carried Out at pH 5.5 and 25 °C[a]

reactant	K_d (mM)	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	k_{off}/k_{on} (mM)
DMS	0.16 ± 0.02	$(1.9 \pm 0.1) \times 10^5$	26 ± 5	0.13 ± 0.04
DMSO	nd[b]	$(4.3 \pm 0.3) \times 10^2$	47 ± 9	109 ± 28

[a] Parameters were obtained as described in the text, from the data depicted in Figure 5. [b] Value not determined.

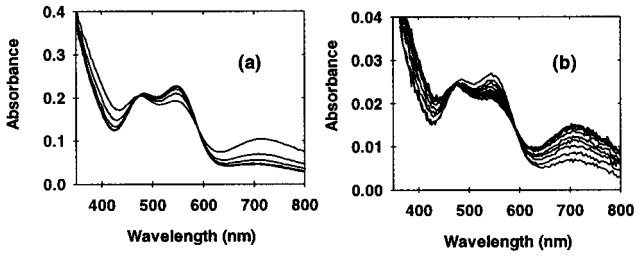


FIGURE 6: Rapid-scan stopped-flow traces showing (a) the reduction of DMSOR by DMS and (b) its subsequent reoxidation by DMSO. For both graphs a and b, spectra were recorded at 2.6 ms intervals starting 1.2 ms after mixing. Thus, the initial spectrum is not that of the resting-state enzyme in graph a or that of the reduced enzyme in graph b. In graph a, DMSOR was mixed with DMS to a final concentration of 10 mM in 25 mM MES at pH 5.5 containing 1 mM EDTA. The final enzyme concentration was 80 μM. In graph b, the enzyme in the same buffer was reduced with 0.4 mM DMS for a few minutes and then mixed with DMSO to a final concentration of 100 mM. The final enzyme concentration was 25 μM.

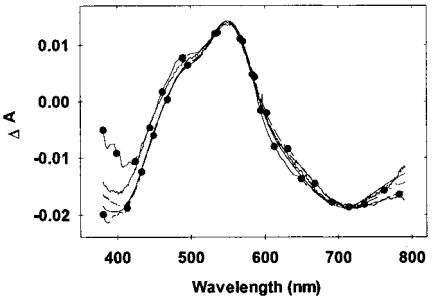


FIGURE 7: Absorption difference spectra for the reduction of DMSOR by DMS and for its reoxidation by DMSO. Data from two reduction experiments (curves with dots, the spectrum of the enzyme shortly after DMS addition minus the spectrum of the resting-state enzyme−DMSO complex) and from three reoxidation experiments (curves without dots, the spectrum after DMS addition minus the spectrum shortly after subsequent DMSO addition). Manual mixing (—) or stopped-flow (- - -) was employed. Before subtraction, spectra were scaled to correspond to the same enzyme concentration. The difference spectra illustrated have been further scaled so they coincide at 550 and 710 nm. Difference spectra obtained for reoxidation had lower amplitudes than the corresponding ones on reduction, because of the incompleteness the reoxidation. Thus, relative to the corresponding reductions, scaling factors of 1.60 and 1.83 have been applied to the illustrated manual and stopped-flow reoxidation spectra, respectively. All experiments were carried out in 50 mM Tris-Cl^- and 1 mM EDTA at pH 8.0. Reduction was achieved with 10 or 60 mM DMS and reoxidation with 200 mM DMSO. In the stopped-flow experiments, the reduction time was 100 ms; for the reoxidation, this was followed immediately (sequential mixing mode) by reoxidation for 10 s.

indicates that under these conditions, the formation of the enzyme−substrate complex is rate-limiting. A linear fit of these data predicts k_{on} and k_{off} values of $(1.9 \pm 0.1) \times 10^5$ M^{-1} s^{-1} and 26 ± 5 s^{-1}, respectively (Table 2). The fraction of DMSOR reduced is also dependent on the DMS concentration. A dissociation constant of 0.16 ± 0.02 mM predicted from a Michaelis−Menten fit of the amplitude data (Figure 5b) is in good agreement with that calculated from the two rate constants (0.13 ± 0.04 mM). Complementary scanning stopped-flow experiments (Figure 6a) showed clear isosbestic points, and analysis (not shown) of the time course indicated a simple exponential. This is consistent with a simple concerted two-electron reduction of the enzyme with no intermediate species.

Stopped-flow studies have also been used to investigate the reoxidation of DMS-reduced DMSOR by DMSO. At pH 8.0, a rate constant of 61 ± 4 s^{-1} was observed. Variations in the DMSO concentration between 4 and 50 mM had no observable effect on the rate or the extent of reoxidation. However, at pH 5.5, both were dependent on the DMSO concentration (Figure 5c,d). A linear fit of k_{obs} for the reoxidation reaction against DMSO concentration gave a k_{on} of $(4.3 \pm 0.3) \times 10^2$ M^{-1} s^{-1} and a k_{off} of 47 ± 9 s^{-1} (Table 2). While the amplitude data (Figure 5d) exhibited some dependence of the amplitude change on the DMSO concentration, the errors do not allow a meaningful determination of the dissociation constant. However, values from the kinetic plot (k_{off}/k_{on}) suggest a dissociation constant of 109 ± 28 mM. As in the reduction experiments, complementary scanning stopped-flow oxidation experiments both at pH 5.5 (Figure 6b) and at pH 8.0 (*37*) showed clear isosbestic points and indicated a simple two-state exponential model, again consistent with a concerted two-electron oxidation of the enzyme not involving any intermediate species. Nevertheless, the incompleteness of the reoxidation is shown by the considerably smaller maximum absorbance change in Figure 5d in comparison with that in Figure 5b.

Incompleteness of the Reoxidation by DMSO of DMSOR Reduced by DMS. We carried out a number of further experiments in an effort to understand the origin of the incompleteness, noted above, of the reoxidation by DMSO of DMSOR reduced by DMS. In different experiments, the extent of this reoxidation varied from as low as 40% to as high as 80%. We postulated that brief treatment as described above of DMSOR with DMS results in two reduced species, one of which can be reoxidized with DMSO while (as demonstrated in several experiments; data not shown) both are reoxidized in the presence of molecular oxygen after removal of DMS by gel filtration. To obtain further information, we compared difference spectra corresponding to the reduction of DMSOR by DMS with those corresponding to its subsequent reoxidation by DMSO. Data are summarized in Figure 7. This includes spectra obtained from manual mixing experiments when the enzyme was reduced for about 5 min prior to the addition of DMSO, and from sequential mixing experiments using the stopped-flow apparatus, when the enzyme was reduced for exactly 100 ms only. The spectrum of DMSO-bound rather than resting-state DMSOR was used in constructing these plots so we could focus on changes in the spectrum caused by reduction and reoxidation, and not those resulting from DMSO binding (cf. Figure 3). After appropriate scaling, such reduction and reoxidation difference spectra are indistinguishable from one another within the noise (Figure 7). Thus, if there are two reduced species, we are unable to distinguish them spectrophotometrically. The scaling factors for the manual and stopped-flow experiments in Figure 7 correspond respectively to 63 and 55% reoxidation.

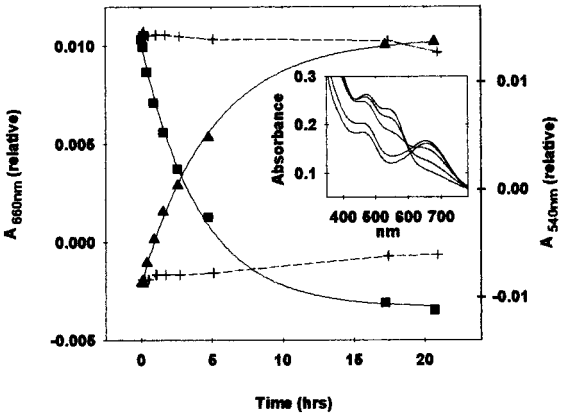


FIGURE 8: Time course of extended aerobic and anaerobic reaction of DMSOR with DMS. The enzyme (40 μM) was treated aerobically or anaerobically with DMS (100 mM) at 25 °C in tightly stoppered spectrophotometer cells, and spectra were recorded at intervals (see Materials and Methods for details of the anaerobic procedures). The experiment was carried out in 50 mM Tris-Cl^- buffer containing 1 mM EDTA at pH 8.0. Plots of relative absorbances, defined as $A_{660} - (A_{630} + A_{690})/2$ [aerobic (▲) and anaerobic (+), lower data set] and $A_{540} - (A_{510} + A_{570})/2$ [aerobic (■) and anaerobic (+), upper data set], vs time are shown. The solid lines through the experimental points are fitted curves for a first-order reaction with rate constants of 0.20 ± 0.01 and 0.25 ± 0.02 h^{-1}, respectively. The inset shows spectra recorded at the following time intervals after DMS addition (in order of decreasing absorbance at 540 nm): 0.4, 1.6, 4.8, 17.2, and 20.7 h. Though well-defined isosbestic points were not observed, probably because of changing turbidities, the procedure used in the experiment whose results are depicted in the main graph permitted meaningful data analysis.

Effect of Oxygen in the Slow Phase of the Reaction of DMS with DMSOR. As stated above, brief treatment of DMSOR with DMS was fully reversible, as shown by complete restoration of the resting enzyme's spectrum on aerobic gel filtration. However, there were slight spectral changes, with shifts of the 720 nm peak toward shorter wavelengths, if the exposure to DMS was a little more prolonged (data not shown). Systematic investigation of this phenomenon was handicapped both by evaporation of DMS from solution and by the development of turbidities in its freshly prepared solutions (see Materials and Methods). Furthermore, the changes were found to be oxygen-dependent, necessitating the use of anaerobic techniques. As shown in Figure 8, upon much more prolonged aerobic treatment of DMSOR with DMS, there is a pronounced change in the UV−visible absorption spectrum. Turbidities, which apparently changed during the course of the reaction, complicated the experiment. Despite this, a kinetic analysis was performed, as illustrated, that minimized interference from this cause. The changes occurring at 660 and 540 nm are consistent in indicating a first-order reaction with rate constants of 0.20 ± 0.01 and 0.25 ± 0.02 h^{-1}, respectively, as deduced from the theoretical curves in Figure 8. The spectral change in this slow reaction phase, unlike that occurring in the initial phase, was not reversed when the sample was gel filtered. Thus, the spectrum of the enzyme at the conclusion (22 h reaction time) of the experiment of Figure 8 changed little on gel filtration, yielding curve d in Figure 4. The enzyme form so produced will be referred to as "DMSOR modified by DMS", abbreviated $DMSOR_{mod}$. While this material was fully active in the $MV^•$:DMSO assay (Table 1), it was almost completely inactive in the DMS:PMS/DCPIP assay [2.5% activity remaining (Table 1), compared with 98.8−99.6% completion of the spectral modification reaction, as calculated from the theoretical plots in Figure 8]. Note that quantitative conversion of the enzyme to $DMSOR_{mod}$ was achieved only in experiments performed in vessels that were tightly sealed to prevent loss of DMS. The spectrum of $DMSOR_{mod}$ was not changed by the addition of DMSO (data not shown). However, this form was found to revert to the resting enzyme form, when treated with $MV^•$, dithionite, and DMSO (the conditions of the forward assay) and then gel filtered into 50 mM Tris-Cl^- and 1 mM EDTA at pH 8.0. Both the original spectrum and most of the enzymatic activity were restored by this treatment (Table 1, $DMSOR_{regen}$).

A requirement for oxygen as well as DMS for conversion of DMSOR to $DMSOR_{mod}$ is clearly shown (Figure 8) by the anaerobic experiment carried out in parallel with the aerobic one described above. The magnitude of the spectral changes observed was some 10-fold smaller than those in the aerobic experiment. It seems reasonable to assume that had more rigorous anaerobiosis been achieved (see Materials and Methods) the reaction would have been completely suppressed.

DISCUSSION

Assay Procedures, Properties of the Enzyme, and Steady-State Kinetic Parameters. Conflicts in the literature in relation to the structure of DMSOR make it imperative that samples of the enzyme used for physical studies be carefully characterized and assayed. Previously, workers have assayed the enzyme in the forward direction only, with $BV^•$ or $MV^•$ as the reducing substrate and DMSO as the oxidizing substrate. The procedures we have introduced represent an improvement in both the convenience and the precision of this assay. In addition, to provide a further parameter relating to the activity of the enzyme, we have for the first time used also the reverse assay with DMS as the reducing substrate and the mixed dyes, PES and DCPIP, as oxidizing substrates. For this work, we used four enzyme batches prepared in three different laboratories. We were not able to distinguish the properties of these batches either in absorbance ratios or in specific activities in either assay (Table 1). Minor differences in the absorption spectra were largely ascribed to light scattering. Unfortunately, meaningful comparison of our data with specific activities in the literature is not easy, because of the different conditions that were used, or in some cases because of the conditions that were not specified. Also, and this may be particularly significant, methods of determining the enzyme concentration varied greatly. Our specific activities are comparable to those of McEwan et al. (*38*), if a reasonable temperature coefficient is assumed, but are rather lower than those of McEwan et al. (*24*). We were unable to compare our data with those of Bastian et al. (*25*) since they do not appear to specify the temperature of their assay (see also refs *35* and *46*).

The form of the progress curves in $MV^•$:DMSO assays, with the rate accelerating progressively as the viologen radical is oxidized, which we attribute to inhibition of the enzyme by excess viologen radical, has not been reported by earlier workers. The precise mechanism of such inhibition

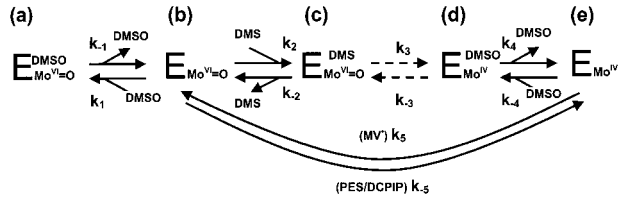
is not certain. Most simply, it could perhaps involve some form of over-reduction of the enzyme molecule by reaction with a further viologen radical. This could involve loss of an oxygen ligand from the metal. Alternatively, reduction of the pterin to the tetrahydro state could be involved, though this could only occur after opening of the pyran ring. In any case, such a picture is complicated by the requirement of the enzyme, during turnover, for two electrons from the reaction of two successive radicals. Dimerization of the radicals at high concentrations is well-established (*47*, *48*) and may play a part in the observed inhibition. Overall, and including data at high MV_{ox} concentrations, it seems that more than one inhibition mechanism contributes to the observed phenomena. The low values derived from these data (Table 1) of the apparent Michaelis constant, K_s, for both dyes and of the inhibition constant, K_i, particularly for $BV^{\bullet}$ and especially at low pH values, are noteworthy.

Our data on the effect of pH on the activity of the enzyme supersede earlier work (*25*) which appeared to indicate that it had very little activity in the $BV^{\bullet}$:DMSO assay at pH 5.5. This discrepancy may be due to the Bastian et al. having failed to take account of the inhibition phenomenon described in this work. Full activity of the enzyme at low pH values adds to the relevance of the crystal structures determined under such conditions (*11*).

The low activity of the enzyme below pH 7 in the reverse assay with DMS as the reducing substrate is presumably related to the redox potentials of the dyes that were used. The potential of the DMS−DMSO couple is 160 mV at pH 7.0 (*45*). For DCPIP, the corresponding value is 57 mV higher than this, at 217 mV (*49*), consistent with the reaction proceeding in this direction. Thus, the requirement for PES with a reported potential of 55 mV (*49*) is difficult to understand in terms of redox potentials, though its ability to act as a one-electron oxidant may be crucial. The DMS−DMSO potential is expected to increase by 59 mV per unit fall in pH, while there is a rather larger rate of increase for the DCPIP potential. The corresponding data for PES are apparently not available, though if this potential was less pH-dependent, this could explain the rapid decrease in the activity of the enzyme at pH values below the optimum value at pH 8.3.

Rates of Individual Steps of the Catalytic Cycles of DMSOR, for the Forward (DMSO Reduction) and Backward (DMS Oxidation) Reactions. Scheme 1 summarizes the conclusions from stopped-flow and other experiments. Rate constants in Scheme 1 may be deduced as follows. k_{-1}/k_1 is 17 mM which is derived from the data for DMSO binding to the resting enzyme and corresponds to the dissociation constant for this dead-end complex. While at pH 8.0 the rate of DMSOR reduction by DMS is too rapid for detailed study, at pH 5.5 accurate rates can be measured, and under the conditions that were used, formation of a protein−DMS complex is rate-limiting. From the single-wavelength stopped-flow studies, the rate constants at pH 5.5 for the association and dissociation of DMS with the oxidized enzyme (Table 2, k_{on} and k_{-off} for DMS) correspond in Scheme 1 to a k_2 of 1.9×10^5 M^{-1} s^{-1} and a k_{-2} of 26 s^{-1}. The corresponding rate constants for the association and dissociation of DMSO with the reduced enzyme (Table 2, k_{on} and k_{-off} for DMSO) similarly correspond to a k_{-4} of 4.3×10^2 M^{-1} s^{-1} and a k_4 of 47 s^{-1}. At this pH value, the rates of the catalytic step (k_3 and k_{-3}) must be greater than the observed rates at the highest substrate concentrations that were used (Figure 5a,c); i.e., $k_3 > \sim 220$ s^{-1}, and $k_{-3} > \sim 70$ s^{-1}, respectively. The values for k_4 and k_{-4} are calculated from a linear fit (Figure 5c). If at higher DMSO concentrations the rate of the catalytic step were being approached, the observed rates would saturate. There are indications in the data that this is happening, suggesting that the catalytic step (k_{-3}) is close to the maximum observed rate of 65 s^{-1}. At pH 8.0, k_{-3} was measured directly as 61 s^{-1}. Thus, for the reoxidation, the formation of the protein−DMSO complex is rate-limiting at pH 5.5, while at pH 8.0, a catalytic step (k_3 and k_{-3}) is limiting. Conversely, there is a dramatic increase in the overall rate of the reduction reaction on raising the pH from 5.5 to 8.0. Presumably, this is due to an increase in k_2. Thus, it appears that, on changing from pH 8.0 to 5.5, there has been a decrease in the rate of the interaction of DMSOR with both DMS and DMSO, while the rate of the catalytic step is largely unaltered, or faster.

Scheme 1: Catalytic Cycles of DMSOR, for the Forward (DMSO Reduction) and Backward (DMS Oxidation) Reactions[a]



[a] This scheme summarizes the conclusions from stopped-flow experiments carried out at pH 5.5 (Table 2) and additional experiments carried out at pH 8.0 as well as data for DMSO binding to the resting-state enzyme and from measurements of the catalytic center activity (Table 1). Species c and d are Michaelis complexes, and species a is a dead-end complex. Reactions with dyes in the assays, controlled by k_5 and k_{-5}, both involve two successive one-electron reactions. The catalytic step, k_3 and k_{-3}, involves transfer of an oxygen atom and a two-electron Mo(VI)−Mo(IV) interconversion. Note that though the transferred oxygen atom (*21*) is depicted as an oxo ligand of molybdenum, this point may not be fully established. For rate constants of the individual steps, see the text.

It is noteworthy that the reactions of the catalytic step, k_3 and k_{-3}, both occur at rates considerably faster than the enzyme turnover in either the forward or the backward assay (Table 1). Thus, the one-electron reactions with the artificial dyes (k_5 and k_{-5} in Scheme 1) used in the steady-state assays must be rate-limiting. This finding necessitates reinterpretation of the conclusions of Bennett et al. (*32*). These workers unsuccessfully used EPR to seek a Mo(V) species during steady-state enzyme turnover of DMSO and $MV^{\bullet}$. This negative result was interpreted as indicating that reaction with DMSO was rate-limiting. The alternative, that the second reaction step with $MV^{\bullet}$ (part of k_5 in Scheme 1) is faster than the first, now seems more likely. It is interesting that for the two-electron reactions, the rate of DMS oxidation by DMSOR is faster than that of DMSO reduction. This is true at both pH 5.5 and 8.0. The enzyme thus appears to be kinetically more effective as a DMS-oxidizing enzyme than as a DMSO reductase. Whether this finding is significant in relation to the evolution of DMS-oxidizing (*43*) and DMSO-reducing enzymes is uncertain.

Our investigations are important in establishing for the first time the catalytic competence of the DMS-reduced

enzyme species, the characteristic UV−visible spectrum of which was first reported by McAlpine et al. (*13*). The kinetic data suggest that the binding and subsequent reaction of DMS with the oxidized, and of DMSO with the reduced, enzyme species are fully reversible and that these reactions occur by simple two-electron concerted processes. Yet not all the oxidized form is recovered in the reoxidation experiments. This incompleteness of the reoxidation reaction is discussed below.

Extent of the Reduction of DMSOR by DMS and of Its Reoxidation by DMSO and the Incompleteness of the Latter Process. Precise quantitation of the extent of reduction of DMSOR by DMS and of its subsequent reoxidation by DMSO is made difficult by the volatility of DMS. No doubt further work would be facilitated by the use of closed systems in conjunction with a reliable assay for DMS [e.g., GC−MS (*50*)]. Nevertheless, our data indicate that reduction is a simple one-step process. Thus, the experiment whose results are depicted in Figure 5b at pH 5.5 is consistent with reduction being governed by a simple redox-controlled equilibrium between the DMS−DMSO couple and that for the two-electron reduction−two-electron reoxidation of the enzyme. The data indicate that the potential for the enzyme is lower by about 40 mV than that of substrate. Limited data from additional experiments (not shown) at pH 8.0 suggested a similar or slightly lower value for the potential difference.

In contrast to this simple situation, reoxidation by DMSO of enzyme reduced by DMS is always incomplete even when a very large excess of DMSO is used. Furthermore, the extent of the reoxidation is somewhat variable. The incompleteness has been observed for different batches of enzyme and both with manual mixing and in stopped-flow experiments. In the latter, the exposure to DMS before DMSO addition was limited to 100 ms, restricting the possibility of complications arising from any slower side reactions with DMS (such as, e.g., the formation of a hypothetical precursor of $DMSOR_{mod}$). For samples reduced briefly with DMS by manual mixing, and then partially reoxidized with DMSO, full reoxidation could, however, be achieved if the protein was subsequently gel filtered into buffer. Thus, there must be at least two reduced enzyme species, one that can be reoxidized by DMSO and the other not, while both can be fully reoxidized by molecular oxygen. However, despite considerable effort, we were unable to distinguish spectroscopically between the DMSO reoxidizable and the nonreoxidizable enzyme forms. Thus, the difference between them must be a subtle one, and further work perhaps utilizing other spectroscopic methods will be required to elucidate its nature. It could be due an impurity in or produced from the DMS or to an inhibitor in the DMSOR preparations, or to the presence of some modified but catalytically competent form or forms of the resting enzyme.

Nature and Significance of the New DMSOR Form Generated on Extended Treatment with DMS in the Presence of Air. The nature of the change brought about in the enzyme by prolonged treatment with DMS under aerobic but not anaerobic conditions and yielding $DMSOR_{mod}$ is of considerable interest. It involves a pronounced modification of the UV−visible spectrum and the loss of all activity in the reverse assay. Though activity in the forward assay is apparently not affected, this does not exclude the possibility that $DMSOR_{mod}$ might be inactive toward the in vivo reductant, which undoubtedly has a higher redox potential than $MV^{\bullet}$. This point is uncertain, since the normal enzyme is regenerated by treatment of $DMSOR_{mod}$ with $MV^{\bullet}$ and DMSO, as in the assay.[4]

Existence of the new form is relevant to the structure of DMS-reduced enzyme as determined by EXAFS (*34*), by crystallography (*13*), and from Raman spectroscopy (*35*). The question must arise in each case as to whether the form that was studied was wholly the initially formed species or whether some conversion to $DMSOR_{mod}$ had occurred, a possibility that would at best complicate interpretation of the data. In the crystallographic work of McAlpine et al. (*13*), the crystals, sealed in a capillary, appeared to retain at the end of the data collection (S. Bailey, unpublished work) the characteristic pink color of the initially formed species, suggesting that little conversion had occurred. On the other hand, in relation to the resonance Raman data, the rather noisy UV−visible spectrum of Garton et al. (*35*) of the DMS-reduced enzyme is obviously different from that of Figure 4 (curve b), but it is not immediately clear whether this is due to turbidity, to loss of DMS, or to partial conversion to $DMSOR_{mod}$.

Until crystallographic and EXAFS data are available, we can only speculate about the structure of $DMSOR_{mod}$. To do this we use, as our starting point, the seven-coordinate structures[5] of the oxidized (*11*, *14*) and DMS-reduced enzyme forms (*13*; notwithstanding the possible reservations about the latter noted above). We also note the conditions under which the new species is formed and reverts to the normal enzyme. A further increase in the coordination number of molybdenum in the normal resting enzyme by addition of oxygen to the metal is presumably unlikely. Thus,

[4] Two papers [George et al. (*51*) and Hilton et al. (*52*)] on closely related work appeared shortly after this paper was submitted for publication. The second of these describes studies of recombinant *R. sphaeroides* DMSOR expressed in *E. coli* and the former investigations by EXAFS of the structures of different forms of the recombinant enzyme. Hilton et al. (*52*) isolated the recombinant enzyme in a form that was different from the wild-type enzyme but that could, by a simple treatment, be converted into one that closely resembled it. The UV−visible spectrum of the "as prepared recombinant enzyme" of these workers (λ_{max} = 650 and 470 nm) shows clear similarities to that of our $DMSOR_{mod}$ (λ_{max} = 660 and 466 nm). Furthermore, the process described by us as "regeneration" of the resting-state enzyme from $DMSOR_{mod}$, involving treatment of $MV^{\bullet}$-reduced enzyme with DMSO, seems essentially identical to the "redox cycling" process employed by them in converting their recombinant enzyme to a form closely resembling the wild-type enzyme. However, it is clear both from the method of preparation and from a comparison of the spectra (noting particularly the much deeper minimum at around 540 nm in the spectrum of our material in Figure 4 and the complete absence in it of the very prominent feature at 380 nm) that the "as prepared recombinant enzyme" of these workers is undoubtely a mixture of species.

[5] In a paper that appeared shortly after this paper was submitted for publication (see also footnote 4), George et al. (*51*) dismissed proposals that DMSOR is seven-coordinate on the basis of various technical criticisms of the EXAFS studies of Baugh et al. (*34*). It is true that EXAFS is a technique that is in general not well suited to the determination of coordination numbers for metals in metalloproteins, particularly for high coordination numbers. However, George et al. (*51*) went on also to dismiss the crystallographic data of McAlpine et al. (*11*, *13*) for seven-coordination in the enzyme. The published electron density maps of these workers would be difficult to interpret in terms of anything other than a seven-coordinate molybdenum in DMSOR, a finding that has now been confirmed independently in the closely related enzyme trimethylamine *N*-oxide reductase from *Shewanella massilia* (*14*).

in principle, reaction with oxygen following reduction by DMS might involve an increase in the oxidation state of the metal, oxidation of the reduced pterin cofactor, oxidation of dissociated thiolate ligands, or some combination of these. Perhaps it is most reasonable to assume that the metal is present in $DMSOR_{mod}$ as Mo(VI) and that a pair of dithiolene sulfurs have dissociated from the metal and become converted to a disulfide.[6] In this state, the enzyme would be locked, with only one pterin coordinated to the metal, though the changes are presumably ones that could be reversed by appropriate reduction, as with $MV^{\bullet}$. Such a structure is analogous to earlier crystallographically determined structures (*8*, *9*), not confirmed in later work (*11*, *14*), that may thus represent degraded enzyme forms. EPR data for the enzyme (*32*) that gave the first evidence of multiple DMSOR forms are also relevant. A reduced DMSOR species is known to give the (desulfo xanthine oxidase-like) Low-*g* split EPR signal. The extreme similarity of the EPR parameters of these species from the two enzymes, in conjunction with the crystallographically derived structure (*7*) of the desulfo form of enzymes of the xanthine oxidase family, provides clear evidence (*32*, *33*) that in the species giving rise to this signal from DMSOR, only two MTP sulfur atoms are coordinated. Though this adds further plausibility to the proposed structure for $DMSOR_{mod}$, additional work is needed to establish the relationship of the latter to the species giving the Low-*g* split signal.[7] Evidence that thiolate oxidation can readily occur in DMSOR species is provided by recent work by McEwan and co-workers (*53*). According to this study, treatment of DMSOR with dithionite under appropriate conditions yields a thiyl radical species, detected by EPR and centered on one of the MPT thiolate sulfur atoms, no longer coordinated to Mo. Such a species, presumably arising from some type of radical-mediated damage to reduced DMSOR, could obviously be a precursor for a further species having both sulfurs of one MPT dissociated and formed into a disulfide, as in our proposed structure of $DMSOR_{mod}$.

ACKNOWLEDGMENT

We thank Mr. Stephan Lamart for assistance with preliminary experiments.

[6] As already noted (see footnotes 4 and 5), the form of DMSOR described as the "as prepared recombinant enzyme" and investigated in a recent paper by George et al. (*51*) is clearly related to our $DMSOR_{mod}$ but undoubtedly has a lower purity. In contrast to our suggestions about the possible structure of $DMSOR_{mod}$, George et al. (*51*) have concluded from EXAFS investigations that in their material, molybdenum is coordinated by two oxo ligands at 1.72 Å and four sulfurs at 2.48 Å. However, how such a structure might arise from reaction of molecular oxygen with any likely structure that might be postulated for DMS-reduced native enzyme is difficult to understand. We conclude that further structural work with $DMSOR_{mod}$ is urgently needed to resolve matters.

[7] The reason for the variability in the work of Bennett et al. (*32*) in the extent of conversion by $Na_2S_2O_4$ of DMSOR samples to the Low-*g* Type 1 EPR signal-giving species has not been established. However, the possibility that oxygen-mediated damage to a reduced enzyme form may be involved in the conversion, as presumably is the case in the formation of $DMSOR_{mod}$, is suggested by early experiments (N. A. Turner, A. G. McEwan, and R. C. Bray, unpublished work). In these, it was found that this signal developed only after a number of successive small additions of $Na_2S_2O_4$ to the enzyme under semianaerobic conditions.

REFERENCES

1. Rajagopalan, K. V., and Johnson, J. L. (1992) *J. Biol. Chem. 267*, 10199−10202.
2. Hille, R. (1996) *Chem. Rev. 96*, 2757−2816.
3. Kisker, C., Schindelin, H., and Rees, D. C. (1997) *Annu. Rev. Biochem. 66*, 233−267.
4. Enemark, J. H., and Garner, C. D. (1997) *J.Biol. Inorg. Chem. 2*, 817−822.
5. McMaster, J., and Enemark, J. H. (1998) *Curr. Opin. Chem. Biol. 2*, 201−207.
6. Romão, M. J., Archer, M., Moura, I., Moura, J. J. G., Legall, J., Engh, R., Schneider, M., Hof, P., and Huber, R. (1995) *Science 270*, 1170−1176.
7. Huber, R., Hof, P., Duarte, R. O., Moura, J. J. G., Moura, I., Liu, M. Y., Legall, J., Hille, R., Archer, M., and Romao, M. J. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 8846−8851.
8. Schindelin, H., Kisker, C., Hilton, J., Rajagopalan, K. V., and Rees, D. C. (1996) *Science 272*, 1615−1621.
9. Schneider, F., Lowe, J., Huber, R., Schindelin, H., Kisker, C., and Knablein, J. (1996) *J. Mol. Biol. 263*, 53−69.
10. Boyington, J. C., Gladyshev, V. N., Khangulov, S. V., Stadtman, T. C., and Sun, P. D. (1997) *Science 275*, 1305−1308.
11. McAlpine, A. S., McEwan, A. G., Shaw, A. L., and Bailey, S. (1997) *J. Biol. Inorg. Chem. 2*, 690−701.
12. Kisker, C., Schindelin, H., Pacheco, A., Wehbi, W. A., Garrett, R. M., Rajagopalan, K. V., Enemark, J. H., and Rees, D. C. (1997) *Cell 91*, 973−983.
13. McAlpine, A. S., McEwan, A. G., and Bailey, S. (1998) *J. Mol. Biol. 275*, 613−623.
14. Czjzek, M., DosSantos, J. P., Pommier, J., Giordano, G., Mejean, V., and Haser, R. (1998*) J. Mol. Biol. 284*, 435−447.
15. Hille, R., and Massey, V. (1985) in *Molybdenum Enzymes* (Spiro, T. G., Ed.) pp 443−518, Wiley-Interscience, New York.
16. Bray, R. C. (1988) *Q. Rev. Biophys. 21*, 299−329.
17. Wootton, J. C., Nicolson, R. E., Cock, J. M., Walters, D. E., Burke, J. F., Doyle, W. A., and Bray, R. C. (1991) *Biochim. Biophys. Acta 1057*, 157−185.
18. Schindelin, H., Kisker, C., and Rees, D. C. (1997) *J. Biol. Inorg. Chem. 2*, 773−781.
19. Holm, R. H. (1990) *Coord. Chem. Rev. 100*, 183−221.
20. Hille, R., and Sprecher, H. (1987) *J. Biol. Chem. 262*, 10914−10917.
21. Schultz, B. E., Hille, R., and Holm, R. H. (1995) *J. Am. Chem. Soc. 117*, 827−828.
22. Lowe, D. J., Richards, R. L., and Bray, R. C. (1998) *J. Biol. Inorg. Chem 3*, 557−558.
23. Howes, B. D., Bray, R. C., Richards, R. L., Turner, N. A., Bennett, B., and Lowe, D. J. (1996) *Biochemistry 35*, 3874−3874.
24. McEwan, A. G., Ferguson, S. J., and Jackson, J. B. (1991) *Biochem. J. 207*, 305−307.
25. Bastian, N. R., Kay, C. J., Barber, M. J., and Rajagopalan, K. V. (1991) *J. Biol. Chem. 266*, 45−51.
26. DosSantos, J. P., Iobbi-Nivol, C., Couillault, C., Giordano, G., and Mejean, V. (1998) *J. Mol. Biol. 284*, 421−433.
27. Treiber, C. A., Rothery, R. A., and Weiner, J. H. (1996) *J. Biol. Chem. 271*, 27339−27345.
28. Simala-Grant, J. L., and Weiner, J. H. (1996) *Microbiology 142*, 3231−3239.
29. Simala-Grant, J. L., and Weiner, J. H. (1998) *Eur. J. Biochem. 251*, 510−515.
30. Benson, N., Farrar, J. A., McEwan, A. G., and Thomson, A. J. (1992) *FEBS Lett. 307*, 169−172.
31. Finnegan, M. G., Hilton, J., Rajagopalan, K. V., and Johnson, M. K. (1993) *Inorg. Chem. 32*, 2616−2617.
32. Bennett, B., Benson, N., McEwan, A. G., and Bray, R. C. (1994) *Eur. J. Biochem. 225*, 321−331.
33. George, G. N., Hilton, J., and Rajagopalan, K. V. (1996) *J. Am. Chem. Soc. 118*, 1113−1117.

34. Baugh, P. E., Garner, C. D., Charnock, J. M., Collison, D., Davies, E. S., McAlpine, A. S., Bailey, S., Lane, I., Hanson, G. R., and McEwan, A. G. (1997) *J. Biol. Inorg. Chem. 2*, 634−643.
35. Garton, S. D., Hilton, J., Oku, H., Crouse, B. R., Rajagopalan, K. V., and Johnson, M. K. (1997) *J. Am. Chem. Soc. 119*, 12906−12916.
36. Zhao, Z. W., and Weiner, J. H. (1998) *J. Biol. Chem. 273*, 20758−20763.
37. Smith, A. T., Bray, R. C., McEwan, A. G., McAlpine, A. S., and Bailey, S. (1998) *Biochem. Soc. Trans. 26*, S211.
38. McEwan, A. G., Wetzstein, H. G., Ferguson, S. J., and Jackson, J. B. (1985) *Biochim. Biophys. Acta 806*, 410−417.
39. Jones, R. W., and Garland, P. B. (1977) *Biochem. J. 164*, 199−211.
40. Thorneley, R. N. F. (1974) *Biochim. Biophys. Acta 333*, 486−487.
41. Mayhew, S. G. (1978) *Eur. J. Biochem. 85*, 535−547.
42. Corbin, J. L., and Watt, G. D. (1990) *Anal. Biochem. 186*, 86−89.
43. Hanlon, S. P., Toh, T. H., Solomon, P. S., Holt, R. A., and McEwan, A. G. (1996) *Eur. J. Biochem. 239*, 391−396.
44. Hanlon, S. P., Holt, R. A., Moore, G. R., and McEwan, A. G. (1994) *Microbiology 140*, 1953−1958.
45. Wood, P. M. (1981) *FEBS Lett. 124*, 11−14.
46. Hilton, J. C., and Rajagopalan, K. V. (1996) *Arch. Biochem. Biophys. 325*, 139−143.
47. Kosower, E. M., and Cotter, J. L. (1964) *J. Am. Chem. Soc. 86*, 5524−5527.
48. Park, J. W., Choi, N. H., and Kim, J. H. (1996) *J. Phys. Chem. 100*, 769−774.
49. Clark, W. M. (1960) in *Oxidation-reduction potentials of organic systems*, Williams and Wilkins, Baltimore, MD.
50. Ginzburg, B., Chalifa, I., Zohary, T., Hadas, O., Dor, I., and Lev, O. (1998) *Water Res. 32*, 1789−1800.
51. George, G. N., Hilton, J., Temple, C., Prince, R. C., and Rajagopalan, K. V. (1999) *J. Am. Chem. Soc. 121*, 1256−1266.
52. Hilton, J. C., Temple, C. A., and Rajagopalan, K. V. (1999) *J. Biol. Chem. 274*, 8428−8436.
53. Lane, I., Hanson, G. R., and McEwan, A. G. (1997) Meeting on molybdenum enzymes, University of Sussex, Brighton, U.K., April 1997, Abstracts.

BI9902034