

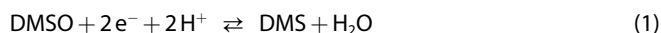
In Vivo Oxo Transfer: Reactions of Native and W-Substituted Dimethyl Sulfoxide Reductase Monitored by ^1H NMR Spectroscopy

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KEYWORDS:

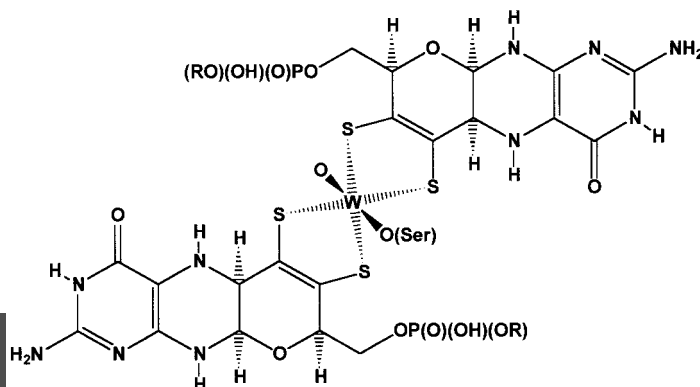
DMSO reductase • enzymes • molybdenum • TMAO reductase • tungsten

Molybdenum enzymes catalyze a wide variety of reactions that involve the net transfer of an oxygen atom either to or from the substrate with the metal cycling between the oxidation states Mo^{VI} and Mo^{IV} .^[1] The periplasmic dimethyl sulfoxide reductases (DMSORs) of the photosynthetic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* function in a respiratory chain with DMSO as the terminal electron acceptor. The DMSORs catalyze the environmentally important reaction (1)^[2, 3] that involves the direct transfer of an oxygen atom from DMSO to Mo^{IV} producing dimethyl sulfide (DMS).^[4]



These enzymes have a high affinity for DMSO and will also catalyze the reduction of trimethylamine-*N*-oxide (TMAO) to trimethylamine (TMA).^[5] Also, the DMSORs from *R. capsulatus* or *R. sphaeroides* (*M_r* ca. 85 000) are important as two of the simplest known molybdenum enzymes, in that they contain only one redox active center, and several crystallographic characterizations of these systems have been reported.^[6, 7] The substitution of molybdenum by tungsten can be accomplished by natural uptake under W-rich, Mo-depleted, conditions, and the protein so produced is completely structurally analogous to the natural enzyme.^[8] Mo- and W-DMSORs comprise one polypeptide chain, enveloping a single prosthetic group that contains the metal bound to two molybdopterin guanine dinucleotides, the oxygen atom of a serine residue and, in the M^{VI} state, an oxo group (Scheme 1).

Activity assays^[9] were accomplished to assess separately the reductive and oxidative components of the catalytic



Scheme 1. Schematic representation of the metal center of oxidized W-substituted DMSO reductase of *Rhodobacter capsulatus* (R = guanine dinucleotide).^[8] For the naturally occurring Mo enzyme, the presence of a third oxygen atom has been debated and this is reviewed by Li et al.,^[6] following earlier reports.^[7]

cycle for the purified proteins and showed that W-DMSOR reduces DMSO approximately $17 \times$ faster than Mo-DMSOR. This result is consistent with the analysis of Sung and Holm of information obtained for Mo and W isoenzymes,^[10] that oxo transfer from the substrate to the reduced metal center is faster for W than for Mo. However, and in stark contrast to Mo-DMSOR, W-DMSOR does not appear to catalyze the oxidation of DMS.^[8] EPR potentiometric titrations have indicated that W-DMSOR has midpoint potentials (vs. SHE) of -203 mV and -105 mV for the $\text{W}^{\text{VI}}/\text{W}^{\text{V}}$ and $\text{W}^{\text{V}}/\text{W}^{\text{IV}}$ couples, respectively.^[8] Each potential is about 325 mV lower than that of the corresponding couple of Mo-DMSOR.^[2] The reduction of DMSOR by ubiquinol in vivo is mediated by the pentaheme *c*-type cytochrome DorC, which has midpoint potentials of -34 , -128 , -184 , -185 , and -276 mV (vs. SHE).^[11] Therefore, DorC should be capable of reducing oxidized W-DMSOR, allowing this protein to turn over inside a cell.

We have assessed the physiological activity of W-DMSOR in vivo by ^1H NMR spectroscopy, by employing the approach of King et al.^[12] and Richardson et al.,^[13] who monitored the turnover of DMSO and TMAO by *R. capsulatus* cells. In our investigations, the Mo- and W-grown cells contained the same amount of DMSOR; presumably, *R. capsulatus* uses the same pathway to incorporate Mo or W from the corresponding $[\text{Mo}_4]^{2-}$ ion. This result contrasts with that of Santini et al., who observed that the amount of tungsten-substituted TMAO reductase produced from *E. coli* by genetic manipulation of the pathway for metal uptake was about 15 % of the level found with molybdenum.^[14]

The ^1H NMR studies accomplished herein have assessed the ability of Mo-DMSOR and W-DMSOR to catalyze oxo transfer from DMSO or TMAO in intact *R. capsulatus* cells. Each of the four species of principal interest exhibited a singlet in the ^1H NMR spectrum, with chemical shifts of: $\delta = 2.73$ (DMSO), 2.11 (DMS), 3.27 (TMAO), and 2.90 (TMA). The DMS signal slightly overlaps the propionate methine signal ($\delta = 2.19$). In each experiment, the initial ^1H NMR spectrum was dominated by the DMSO (or TMAO) singlet, which decreased steadily in amplitude over time,

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with concomitant growth of the DMS (or TMA) signal. As noted by King et al.,^[12] reduction of both DMSO and TMAO produced a second product (X), in parallel with the main reduction; we have identified X for TMAO reduction as Me₂NH (DMA) ($\delta = 2.73$; see Experimental Section).

The time course for the reduction of DMSO and TMAO with turnover of the *R. capsulatus* cells is readily followed by ¹H NMR spectroscopy (see ref.[12] and Figure 1). Plots of concentration of substrate or product versus time (e.g. Figure 2) were essentially

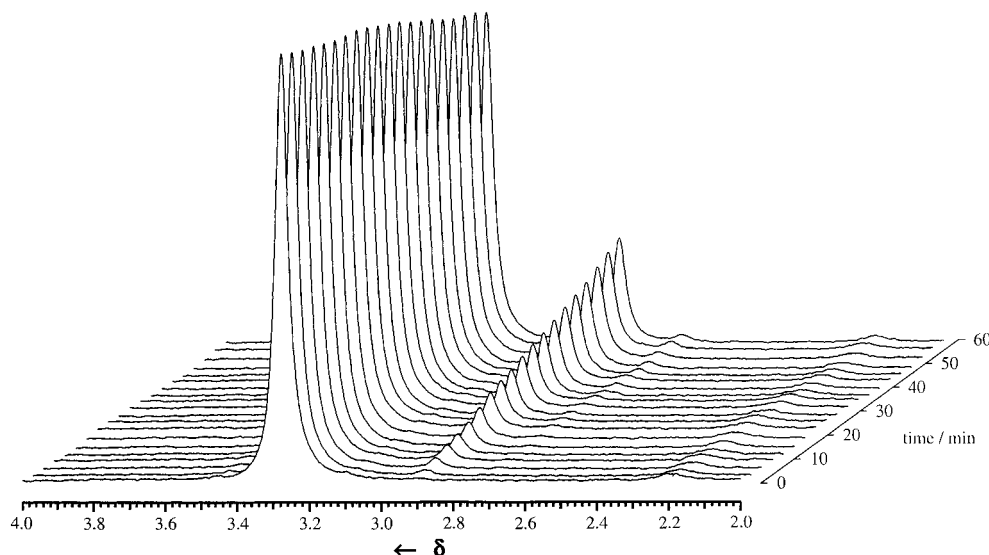


Figure 1. 400 MHz ¹H NMR spectra recorded at 1 minute intervals for the reduction of TMAO by *R. capsulatus* cells grown in a medium containing Na₂WO₄. Lorentzian line-broadening of 3 Hz was used with no reference deconvolution. The resonances are assigned as: $\delta = 3.27$ (TMAO), 2.90 (TMA), 2.73 (DMA), 2.19 (propionate methine).

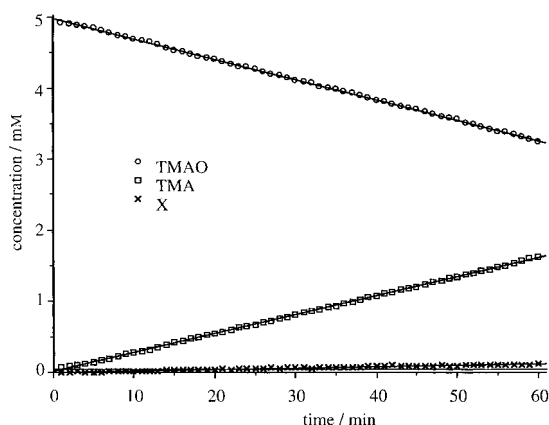


Figure 2. Variations in the relative concentrations of TMAO, TMA, and DMA with time for the experiment conducted as shown in Figure 2. Solid lines show the results of linear regression.

linear, indicating a zeroth-order process and the rates of reactions observed are summarized in Table 1. For the natural system (Mo-grown cells), the values obtained are similar to those of King et al.,^[12] who studied cells suspended in a malate medium rather than a propionate medium as used here. These authors observed that DMSO was reduced by *R. capsulatus* cells

Table 1. Rate ($\text{mM min}^{-1} \times 10^2$) of the appearance and disappearance of the ¹H NMR signals observed in the turnover of DMSO and TMAO by *Rhodobacter capsulatus* cells grown on a medium containing Na₂MoO₄ (3 μM), (Mo) or Na₂WO₄ (3 μM), plus Na₂MoO₄ (6 nM) (W). Estimated experimental errors $\leq 5\%$.

	DMSO	DMS	X	TMAO	TMA	DMA
Mo	− 2.73	2.24	0.494	− 13.0	13.0	0.153
W	− 0.237	0.189	0.048	− 2.86	2.66	0.201

at about 12% of the rate of TMAO reduction; we observed that DMSO was reduced at about 21% of the rate of TMAO reduction for the Mo-grown cells and about 8% for the W-grown cells. The W-grown cells reduce both DMSO and TMAO at a significantly slower rate than the Mo-grown cells; the relative rates are about 9 and 22%, respectively. Nevertheless, the W-grown cells are clearly capable of turnover with DMSO or TMAO as the terminal electron acceptor.

The ¹H NMR studies reported herein provide a further illustration of the utility of this approach to assay the activity of intact cells.^[12, 13] The lack of an ability of isolated W-DMSOR

to catalyze the oxidation of DMS^[8] suggested that this system might not be capable of physiological activity. However, the results obtained in this study clearly demonstrate that W-substituted DMSOR is physiologically active in *R. capsulatus* cells. In nature, tungsten enzymes occur in thermophilic organisms and generally catalyze reactions that have a low redox potential.^[15] Molybdenum enzymes operate at a higher redox potential and are employed by a wide range of organisms that exist at ambient temperature.^[1] Thus, mesophilic *R. capsulatus* cells utilize molybdenum enzymes (e.g. Mo-DMSOR). W-substituted *R. capsulatus* cells turnover at a slower rate than their Mo counterparts, even though the reduction of the substrate by the isolated reduced enzyme proceeds at a significantly (ca. $\times 17$) faster rate for W-DMSOR versus Mo-DMSOR.^[8] Thus, reduction of the oxidized state (M^{VI}) to the reduced state (M^{IV}), where $\text{M} = \text{Mo}$ or W , appears to contain the rate-determining step of the catalytic cycle employed by these enzymes (Figure 3). Crystallographic^[6, 7] and resonance Raman spectroscopic studies of Mo-DMSOR^[16] are consistent with reduction of the Mo^{VI} to the Mo^{IV} state involving the loss of an oxo group; a similar process is expected to occur for the corresponding step in the catalytic cycle of W-DMSOR. This step is expected to proceed at a slower rate for W-DMSOR than for Mo-DMSOR, because: 1) the redox potentials observed for these enzymes^[2, 8] (see above) show that this W^{VI} center is more difficult to reduce than its Mo^{VI} counter-

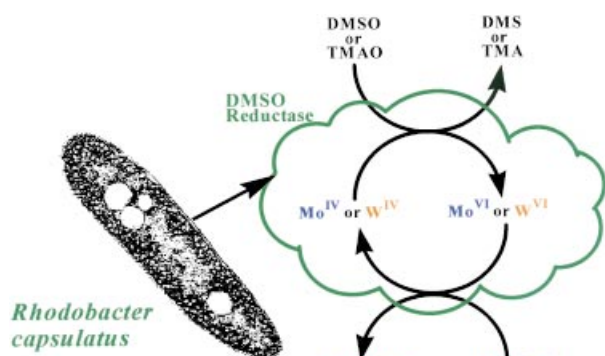


Figure 3. Diagrammatic representation of the catalytic cycle of DMSO reductase in *Rhodobacter capsulatus*, with either Mo or W at the active site, and DMSO or TMAO as the electron acceptor; reduction of the oxidized enzyme by ubiquinol is mediated by the pentaheme c-type cytochrome DorC.

part; and 2) as argued by Tucci et al.,^[17] more energy is required to deform a W–oxo bond than an (equivalent) Mo–oxo bond.

We have shown in this study that W-DMSOR in *R. capsulatus* is physiologically active. However, the rate of turnover of W-DMSOR is significantly slower than that of the native enzyme, Mo-DMSOR. The conversion of the metal from the oxidized (M^{VI}) to the reduced state (M^{IV}), during which the metal loses an oxo group, is considered to contain the rate-determining step of the catalytic cycle employed by these enzymes. The reduction of M^{VI} to M^{IV} proceeds at a significantly slower rate for W-DMSOR than for Mo-DMSOR.

Experimental Section

R. capsulatus cells of strain H123 were grown as described previously^[3] on an "RCV" medium^[18] containing propionate (40 mM) as the carbon source and either Na_2MoO_4 (3 μM), to produce Mo-DMSOR, or Na_2WO_4 (3 μM) plus Na_2MoO_4 (6 nM), to produce W-DMSOR that contained $\leq 1\%$ Mo.^[8] The cells were collected by centrifugation, washed in a low-carbon "RCV" medium that contained propionate (1 mM) and Na_2MoO_4 ($M = \text{Mo or W}$, as appropriate) (3 μM), and recollected by centrifugation. The W culture produced fewer cells (ca. 66% wet weight) than the Mo culture, consistent with the amount of enzyme purified from each culture. Quantification of the cell density, by monitoring the turbidity of the solution by measuring the absorbance at 650 nm, was also consistent with the wet weights recorded. Calibration curves, produced by plotting the weight of dry cells (bacteria harvested by centrifugation and freeze-dried overnight) versus A_{650} , showed that the same weight of cells gave essentially the same A_{650} value for both Mo- and W-grown cells.

^1H NMR spectra were measured at 400 MHz on a Varian INOVA 400 spectrometer using a 5 mm probe equipped with pulsed field gradients of up to 30 G cm^{-1} . A 100 μL solution of each cell-stock suspension (harvested cells added to sufficient of the low-carbon "RCV" medium to produce a stock suspension that contained 0.38 g of cells (wet weight) mL^{-1}); A_{650} was ca. 2.8 in a cuvette of 1 mm pathlength) was mixed with 395 μL of the low-carbon "RCV" medium and the substrate was added (5 μL of DMSO or TMAO (0.5 M) in the low-carbon "RCV" medium). The reaction mixture was placed in an NMR tube with a coaxial capillary containing D_2O and sodium 3-(trimethylsilyl)propionate to provide a field/frequency lock and a

chemical shift reference, respectively. The samples were maintained at room temperature (ca. 20°C), and 60 spectra, each comprising 20 transients acquired over 1 min, were recorded for each experiment, except for the comparatively slow reaction of the W-grown cells with DMSO, for which 180 spectra each of 20 transients s^{-1} were recorded over 1 min, followed by 60 spectra each of 200 transients recorded over 10 min. The WATERGATE pulse sequence^[19] was used with low power water presaturation, giving approximately 10^5 -fold suppression of the H_2O signal. Over the time scale of the experiments, some line-shape drift occurred and was corrected where necessary using the FIDDLE algorithm for reference deconvolution.^[20] Signal intensities were estimated from peak heights, which were normalized to take into account changes in probe tuning and differences in line width and T_1 . DMA was identified by comparison of the ^1H and ^{13}C NMR spectra, recorded for the supernatant from a turnover experiment, with those of an authentic mixture of TMAO/TMA/DMA in the same medium, and by electrospray mass spectrometry. However, mass spectrometry and NMR spectroscopic studies have not allowed the identification of X in DMSO turnover, but have eliminated the demethylated, molecular products MeSH and MeSSMe as possibilities for the singlet at $\delta = 1.93$.^[21] The enzyme dimethyl sulfide monooxygenase is known to catalyze such demethylation reactions in *E. coli*.^[22]

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Studies on DNA Cleavage by Cytotoxic Pyrrole Alkaloids Reveal the Distinctly Different Behavior of Roseophilin and Prodigiosin Derivatives

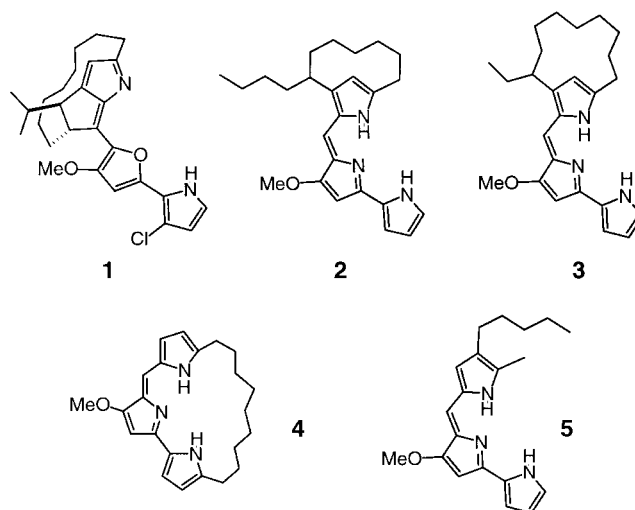
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KEYWORDS:

alkaloids • antitumor agents • DNA cleavage • prodigiosins • pyrroles

Roseophilin (**1**), a secondary metabolite isolated from a culture broth of *Streptomyces griseoviridis*, has attracted considerable attention for the intricate molecular topology of its ansa-bridged azafulvene core as well as for the potent cytotoxicity against various human cancer cell lines.^[1, 2] Several conceptually different approaches aiming at the total or partial synthesis of this challenging target have been reported and a set of functional derivatives has been prepared.^[3–5] The biological mode of action of **1**, however, still remains elusive.

The closest structural relatives to roseophilin occurring in nature are the members of the prodigiosin family such as **2–4**.^[6–8] Like **1**, these alkaloids contain an azafulvene motif as well as an ansa bridge, but incorporate a methoxypyrrole rather than a methoxyfuran as the central ring into their heterocyclic perimeter. In view of these structural similarities and the potent cytotoxic and immunosuppressive activities of compounds **2–5** and their congeners,^[9, 10] it seems appropriate to study whether roseophilin (**1**) and the prodigiosins share a common mode of action toward biological receptors. Having access to both series by our total synthesis programs,^[3, 8, 11] we are able to perform



studies along these lines. An interim report comparing the ability of these compounds to damage double-stranded DNA in the presence of metal cations is summarized below.

Very recently, it has been demonstrated that prodigiosin (**5**) binds to DNA and produces oxidative strand cleavage if administered in combination with Cu^{II} salts.^[12] This biological effect is triggered by the formation of π -radical cations through oxidation of the electron-rich pyrrolylpyrromethene chromophore of **5** by the metal cation and may account for the cytotoxicity of this alkaloid.^[13]

By using the same assay system, we were able to gain deeper insights into the structural requirements for effective cleavage of DNA by prodigiosin derivatives. As can be seen from the agarose gel depicted in Figure 1, neither nonylprodigiosin (**4**)^[8b] per se (lane 3) nor Cu^{II} alone (lane 2) damage purified double-stranded plasmid DNA of the bacteriophage Φ X174 (lane 1).^[14, 15] In contrast, a combination of both is very effective: The progress of strand cleavage caused by **4**·Cu(OAc)₂ with increasing incubation time is depicted in lanes 4–13. It is clearly visible that the

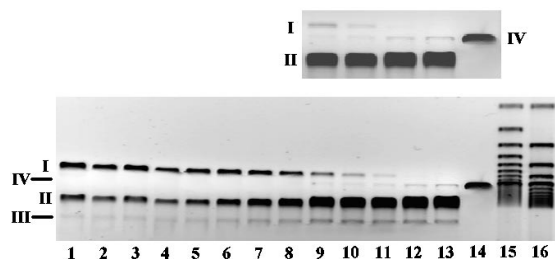


Figure 1. Result of agarose gel electrophoresis showing the extent of DNA cleavage produced by nonylprodigiosin (**4**) in the presence of Cu(OAc)₂ with increasing incubation time at 37°C. Lane 1: DNA alone; lane 2: DNA + Cu^{II}; lane 3: DNA + **4**; lanes 4–13: DNA + **4** + Cu^{II} after the following incubation times: 0 min (4), 5 min (5), 10 min (6), 15 min (7), 20 min (8), 30 min (9), 45 min (10), 60 min (11), 90 min (12), 120 min (13); lane 14: linear DNA formed from scDNA by using the restriction endonuclease XhoI; lane 15: DNA marker (500 base pairs molecular weight difference); lane 16: DNA marker (1000 base pairs molecular weight difference). The insert at the top shows the relevant detail of lanes 10–14 at higher magnification.

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