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Dimethylsulfoxide Reductase: An Enzyme Capable of Catalysis with Either Molybdenum or Tungsten at the Active Site

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Dimethylsulfoxide Reductase: An Enzyme Capable of Catalysis with Either Molybdenum or Tungsten at The Active Site

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Abstract

DMSO reductase (DMSOR) from *Rhodobacter capsulatus*, well-characterised as a molybdoenzyme, will bind tungsten. Protein crystallography has shown that tungsten in W-DMSOR is ligated by the dithiolene group of the two pyranopterins, the oxygen atom of Ser147 plus another oxygen atom, and is located in a very similar site to that of molybdenum in Mo-DMSOR. These conclusions are consistent with W L_{III}-edge X-ray absorption, EPR and UV/visible spectroscopic data. W-DMSOR is significantly more active than Mo-DMSOR in catalysing the reduction of DMSO but, in contrast to the latter, shows no significant ability to catalyse the oxidation of DMS.

Keywords

dimethylsulfoxide reductase, tungsten, crystal structure, X-ray absorption spectroscopy (XAS), pyranopterin

Abbreviations

DCPIP 2,6-dichlorophenol-indophenol
DMS dimethylsulfide
DMSO dimethylsulfoxide
DMSOR dimethylsulfoxide reductase
EPR electron paramagnetic resonance
EXAFS extended X-ray absorption fine structure
Hepes N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
MV methyl viologen
PES phenazine ethosulfate
TMAO trimethylamine *N*-oxide
UV ultraviolet
XAS X-ray absorption spectrum

Introduction

Molybdoenzymes are found in all forms of life from bacteria, through higher plants and animals, to man (Stiefel, 1993). With the notable exception of the nitrogenases, the molybdoenzymes contain a mononuclear molybdenum centre with the metal bound to one or two molecules of a special pyranopterin (Figure 1)(Hilton & Rajagopalan, 1996), originally termed "molybdopterin", which ligates the metal by the sulfur atoms of the dithiolene group Stiefel 1993, Hille 1996. An oxo-group (Mo═O) generally binds to the metal, and other ligands can include a second oxo-group, a sulfido-group (Mo=S), a hydroxo-group (Mo—OH), a water molecule, or a donor atom from the side-chain of an amino acid residue: S^{γ} of cysteine; O^{γ} of serine; or Se^{γ} of selenocysteine Hille 1996, Schindelin et al 1997. The molybdenum centre of all of the molybdoenzymes characterised to date is accessible in the Mo(VI), Mo(V) and Mo(IV) oxidation states and the enzymes all catalyse a conversion, the net effect of which is oxygen-atom transfer to and/or from a substrate. Spectroscopic studies of the molybdoenzymes, notably EPR investigations of the Mo(V) state, have clearly demonstrated that the substrate interacts directly with the metal centre (Bray, 1988). The first direct observation of substrate binding was achieved for the molybdoenzyme DMSO reductase (Mo-DMSOR) from *Rhodobacter* capsulatus by protein crystallography (McAlpine *et al*., 1998).

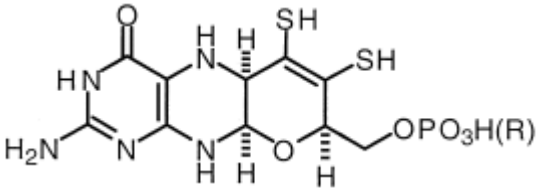


Figure 1. Structure of the pyranopterin, one or two of which ligate the molybdenum or tungsten in molybdo- or tungstoenzymes, respectively. The phosphate group may be bound to a nucleotide; in DMSOR this is a guanine dinucleotide.

The atomic and ionic radii and the chemical properties of tungsten are very similar to those of molybdenum (Greenwood & Earnshaw, 1984). Evidence for the involvement of tungsten in biological systems has only been obtained relatively recently Johnson et al 1996, Hagen and Arendson 1998 and some striking parallels between the nature and function of molybdenum and tungsten centres in enzymes have emerged. The first structural characterisation of "molybdopterin" was obtained by the protein crystallographic investigation of the tungsten enzyme, aldehyde oxidoreductase from *Pyrococcus furiosus*, in which the metal ion is ligated by the dithiolene groups of two pyranopterins (Chan *et al*., 1995). Also (with the possible exception of acetylene hydratase), like their molybdenum counterparts, tungsten centres in enzymes catalyse oxygen-atom transfer reactions Johnson et al 1996, Hagen and Arendson 1998. Nevertheless, so far it has proved convenient to group the enzymes that utilise these metals as belonging to distinct classes: molybdoenzymes Stiefel 1993, Hille 1996 and tungstoenzymes Johnson et al 1996, Hagen and Arendson 1998.

Given the chemical similarities between molybdenum and tungsten and the observation that both metals occur in enzymes ligated by the same pyranopterin (Rajagopalan, 1993), the possibility of tungsten substituting for molybdenum and producing an active enzyme has been explored. Bertram *et al*. (1994) showed that tungstate can substitute for molybdate and sustain the growth of *Methanobacterium thermoautotrophicum* and they identified and characterised a tungsten isoenzyme of formylmethanofuran dehydrogenase. However, tungstate does not support the synthesis of active formylmethanofuran dehydrogenase in *Methanosarcina barkeri*(Schmitz *et al*., 1994). Buc *et al*. (1999) successfully substituted tungsten for molybdenum in TMAO reductase and obtained an active enzyme. However, so far only limited comparisons of the nature and properties of a tungstoenzyme and the corresponding molybdoenzyme Johnson et al 1996, Hagen and Arendson 1998 have been made.

As a continuation of our studies of DMSOR from *R. capsulatus*McAlpine et al 1997, McAlpine et al 1998, Baugh et al 1997, we have investigated the possibility of substituting molybdenum by tungsten and explored the nature and catalytic activity of the resultant metalloprotein. One attractive aspect of DMSOR from *Rhodobacter* is that it is the simplest of the molybdoenzymes. DMSOR can be purified as a monomer of relatively low molecular mass (85,000 Da) containing a single redox active centre comprising molybdenum bound to two pyranopterin guanine dinucleotides Hilton and Rajagopalan 1996, McEwan et al 1991.

Results and discussion

R. capsulatus strain H123, was grown phototropically under conditions described (McEwan *et al*., 1991), and the impact of Na_2WO_4 was investigated by monitoring cell density over a period of several days. The results obtained for various concentrations of Na_2WO_4 are shown in Figure 2. Too high a concentration of Na_2WO_4 prevented all cell growth, but the cells grew well at 3 µM Na_2WO_4; however, the presence of a low concentration (6 nM) of Na_2MoO_4 was essential for cell growth. The reason for this requirement of trace molybdate is not clear. In addition to DMSOR, *R. capsulatus* possesses the molybdoenzymes nitrate reductase and xanthine dehydrogenase (Leimkuhler & Klipp, 1999). Molybdate may be required for the biosynthesis of one or both of these enzymes and/or as an activator for the biosynthesis of "molybdopterin". Also, it is possible that molybdate is required to prevent molybdenum and tungsten uptake to toxic levels. The *modABCD* operon, which codes for a molybdate transporter in *Escherichia coli*, is repressed by the ModE gene product. ModE is activated by molybdate but is six times less sensitive to tungstate (Grunden *et al*., 1999). The ModA transporter binds molybdate and tungstate with equal affinity (Imperial *et al*., 1998). Thus,

in the absence of molybdate, tungstate could accumulate to a level sixfold greater than the usual physiological concentration of molybdate.

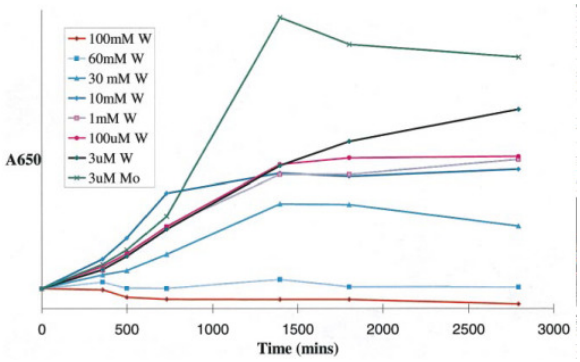


Figure 2. Growth curves for *R. capsulatus* grown phototropically with various concentrations of Na_2WO_4; all media contained a trace of Na_2MoO_4 (6 nM), Kanamycin (25 mg ml^{-1}) and DMSO (45 mM).

Purification of tungsten-substituted DMSOR (W-DMSOR) was achieved using the procedure described for Mo-DMSOR McEwan et al 1991, Bennett et al 1994. The metal content of every preparation of W-DMSOR was measured by ICP mass spectrometry and the W:Mo ratio was found to be >99:1 in each case. In a separate experiment, the growth medium contained equal quantities of the two metals (3 µM Na_2MoO_4 plus 3 µM Na_2WO_4) and the Mo:W ratio in the isolated DMSOR was found to be ca 2:1. Thus the processes of metal uptake, delivery and/or incorporation lead to a significant, preference for the binding of molybdenum versus tungsten in DMSOR of *R. capsulatus*.

Oxidised W-DMSOR was crystallised under the same conditions as oxidised Mo-DMSOR (McAlpine *et al*., 1997) and the crystal structure was determined using data to 2.0 Å resolution (Table 1). The structure of oxidised W-DMSOR, in respect of the polypeptide, the two pyranopterin guanine dinucleotides and the tungsten centre, was found to be very similar to the corresponding details of the Mo-DMSOR structure reported by McAlpine *et al*. (1997). The complete interpretation of the structure at the tungsten was complicated by poorly defined positive difference density in the electron density distribution about the metal. Nevertheless, it is clear that the tungsten is ligated by four dithiolene sulfur atoms, two from each of the pyranopterins (P & Q) with W—S distances in the range 2.4–2.5 Å, and the O^{γ} of Ser147 with a W—O distance of 1.9 Å. The presence of one oxygen ligand, equivalent to O2 in Mo-DMSOR (McAlpine *et al*., 1997), was clearly evident from difference Fourier maps as a well-defined peak at 8.5σ level. This oxygen atom refined to a distance of 1.9 Å from the metal and 2.6 Å from $N^{\varepsilon 1}$ of Trp116. It is not clear whether there is a third oxygen ligand (equivalent to O1 in Mo-DMSOR (McAlpine *et al*., 1997)), since significant positive difference density (6.4σ) is observed close to S1 of the P-pyranopterin. However, this density may be attributed to ripples in the electron density due to series termination errors from the heavy atom (Schindelin *et al*., 1997) or to a degree of heterogeneity in the tungsten environment. Nevertheless, it is clear that the coordination sphere of the metal in W-DMSOR is very similar to that in Mo-DMSOR (McAlpine *et al*., 1997) (see Figure 3).

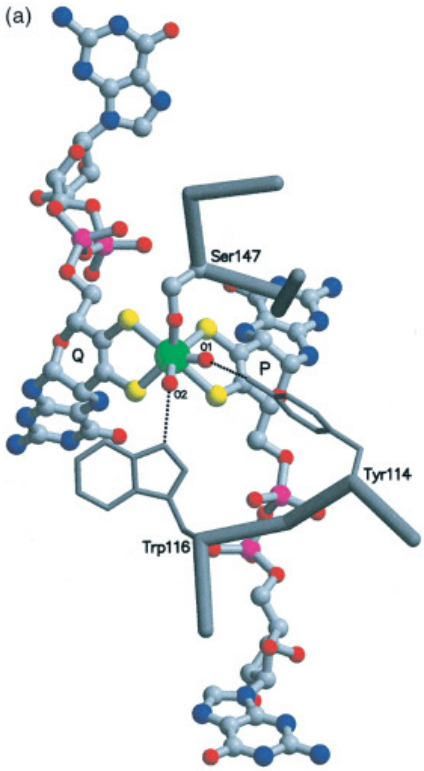
Table 1. Statistics relevant to data collection and refinement

A. *Data collection*	
Resolution (Å)	20–2.0
Number of reflections measured	233,511
Number of unique reflections	51,645
Completeness (%)	98.0
(2.11–2.0 Å)	(92.3)

R_{sym} (%)	6.6
(2.11–2.0 Å)	(15.7)
$\langle I/\sigma I \rangle$	9.0
(2.11–2.0 Å)	(4.2)
B. *Refinement*	
R-factor (20–2.0 Å)	0.146
Free *R*-factor for 5 % of data	0.193
rms deviation bond lengths (Å)	0.011
rms deviation bond angles (deg.)	1.7
Average main-chain temperature factor (Å^2)	17.1
Average side-chain temperature factor (Å^2)	20.1
Average cofactor temperature factor (Å^2)	10.8
Average solvent temperature factor (Å^2)	30.5

$R_{sym} = \Sigma|I(k) - \langle I \rangle|/\Sigma I(k)$ where $I(k)$ and $\langle I \rangle$ represent the diffraction intensity values of individual measurements and the corresponding mean values; s represents the standard deviation.

Crystals for the diffraction experiments were obtained as described for oxidised Mo-DMSOR (McAlpine *et al*., 1997). X-ray diffraction data were collected on station 9.6 of the Synchrotron Radiation Source, CLRC, Daresbury Laboratory, with X-rays at λ = 0.87 Å. Data were processed using the MOSFLM suite of programs (Leslie, 1992) and subsequent processing was done using programs from the CCP4 suite of programs (Collaborative Computational Project No. 4, 1994). The program O (Jones *et al*., 1991) was used for interpretation of electron density maps and model building. Atomic positions and individual atomic temperature factors were refined against the 2.0 Å data with the program REFMAC (Murshudov *et al*., 1997) with weak restraints applied to the metal-ligand distances. The reflections used for calculation of the free R-factor were the same as used for oxidised Mo-DMSOR (McAlpine *et al*., 1997).



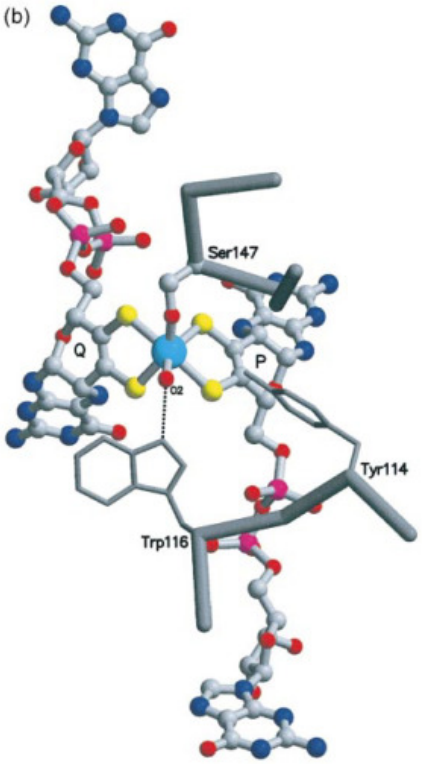


Figure 3. The structure, as determined by protein crystallography, of the metal centre in oxidised DMSOR: (a) Mo-DMSOR, the four dithiolene sulfur atoms and the Mo constitute a square-based pyramid with the Mo at the apex and the three oxygen atoms form a staggered trigonal arrangement above the metal. (b) W-DMSOR, for which significant positive electron density was observed at the position of O1 in Mo-DMSOR (see the text). The W-DMSOR metal site superimposes on the Mo-DMSOR site with an rms deviation of 0.06 Å for all atoms displayed.

To obtain further information about the local coordination environment of the tungsten site in W-DMSOR, the XAS at the W L_{III}-edge was recorded. Figure 4 shows the EXAFS, its Fourier transform, and a simulation of the individual backscattering contributions to the EXAFS. The dominant backscattering arises from a shell of four sulfur atoms at a distance of 2.44 Å from the metal. In addition, weaker but significant backscattering contributions arise from two light atoms (treated as oxygen atoms) at 1.76 and 1.89 Å from the metal. Thus, the information obtained from the W L_{III}-edge EXAFS is consistent with the results of the protein crystallographic study (Figure 3(b)).

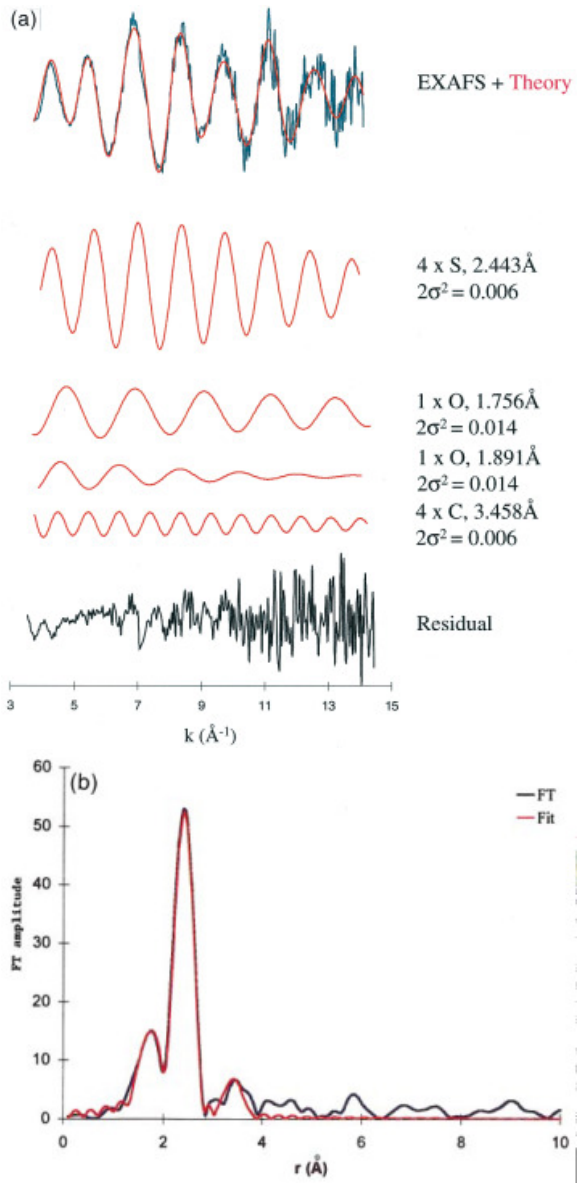


Figure 4. Extended X-ray absorption fine structure (EXAFS) associated with the tungsten L_{III}-edge of W-DMSOR ca 1 mM in Hepes (pH 7.5) at ca 80 K. (a) Normalised EXAFS amplitude (xk^3) showing the individual backscattering contributions. (b) Fourier transform. Blue represents the experimental data, and red the simulated data. The optimum simulation achieved was for: E_0 10,208.8 eV (relative to the edge of a W foil at 10,206.8 eV, each position was taken as the maximum of the first derivative of the absorption edge); backscattering contributions from 4 × S at 2.44 Å ($2\sigma^2$ 0.006 Å^2); 1 × O at 1.76 Å ($2\sigma^2$ 0.003 Å^2); 1 × O at 1.89 Å ($2\sigma^2$ 0.014 Å^2); 4 × C at 3.46 Å ($2\sigma^2$ 0.006 Å^2); R = 30.1. For the XAS (Koningsberger & Prins, 1988), W-DMSO reductase (ca 1 mM in Hepes, pH 7.5) was injected into an aluminium sample cell with Mylar windows, frozen in liquid nitrogen and maintained at ca 80 K throughout the collection of data. The X-ray absorption spectrum at the W L_{III}-edge (9.920–11.050 keV) was recorded in fluorescence mode using a Canberra 30-element solid-state detector on station 16.5 of the Synchrotron Radiation Source, CLRC, Daresbury Laboratory, operating at 2 GeV with an average current of 150 mA. A total of 22 scans were recorded and averaged. The EXAFS was obtained and analysed using the program EXCURV98 (Binsted, 1998). Theoretical fits were generated by adding shells of scatterers to a central tungsten atom and refining the absorber-scatterer distances and their Debye-Waller factors to achieve agreement with the experimental EXAFS and its Fourier transform.

The results of our protein crystallographic and W L_{III}-edge EXAFS studies should be considered in the light of the corresponding studies of Mo-DMSOR, and we note that the determination of the structure of these metal centres provides a considerable challenge for both protein crystallography (Schindelin *et al*., 1997) and EXAFS (George, 1997). Three crystallographic determinations of the structure of oxidised Mo-DMSOR have been reported: one for the enzyme from *R. sphaeroides*(Schindelin *et al*., 1996) and two for the enzyme from *R. capsulatus*McAlpine et al 1997, Schneider et al 1996. Whilst the structure of the proteins and the two pyranopterin guanine dinucleotides is essentially the same in each study, significant differences were reported for the structure at the molybdenum centre. Schindelin *et al*. (1996) found the Mo ion to be bound to the two sulfur atoms of one pyranopterin (P) with the other pyranopterin (Q) being coordinated to the metal by only one sulfur atom; the Mo was also bound to an oxo-group and the O^{γ} of Ser147. Schneider *et al*. (1996) proposed a five-coordinate Mo centre, liganded by the two sulfur atoms of pyranopterin P, two oxo-groups plus the O^{γ} of Ser147. The structure obtained by McAlpine *et al*. (1997)(Figure 3(a)) is significant in two respects. Firstly, this structure is the only one which is in agreement with the conclusions of Mo K-edge EXAFS Baugh et al 1997, George et al 1996, George et al 1999 and resonance Raman (Garton *et al*., 1997) studies of this enzyme that all four sulfur atoms are bound to the metal at essentially the same distance (the EXAFS studies agree on a distance of ca 2.45 Å). Secondly, in addition to an oxo-group and the oxygen atom of Ser147, evidence was obtained for the ligation of a third oxygen atom. However, interpretations of Mo K-edge EXAFS by George et al 1996, George et al 1999) and resonance Raman data by Garton *et al*. (1997) do not require the presence of this third oxygen atom.

W-DMSOR, as isolated, exhibited no EPR signal. Incubation of W-DMSOR with dithionite for one to ten minutes. produced an EPR signal (Figure 5) corresponding to $\leqslant$15(±2) % of the tungsten content. Further incubation with dithionite extinguished the EPR signal. The addition of $K_3[Fe(CN)_6]$ to a solution of as-isolated W-DMSOR elicited no detectable EPR signal at 110 K. These results are consistent with the majority of the tungsten centres in W-DMSOR, as isolated, being present as W(VI) and dithionite reduction producing W(V) and then W(IV). The signal can be simulated at both X and S-band using a unique set of parameters: g_1, 1.966; g_2, 1.932; g_3, 1.887; A_1, 1.28; A_2, 0.84; A_3, 0.94 mT; $\alpha = \beta = \gamma = 0$. These are comparable to those reported for W(V) centres in enzymes Johnson et al 1996, Hagen and Arendson 1998 and involve an $I = 1/2$ W(V)-^{1}H superhyperfine interaction suggestive of a W(V)-OH moiety. This appears to be the first observation of this moiety in a protein. The rhombicity $[(g_1 - g_2)/(g_1 - g_3)]$ and orientation of the W(V) *g*-values are extremely similar to that of the Mo(V) “high-*g* split” signal of Mo-DMSOR Bennett et al 1994, Bastian et al 1991. Furthermore, both the magnitude and orientation of the metal-proton superhyperfine coupling tensors of the two centres are very similar. These observations indicate that the two M(V) centres experience essentially the same ligand field.

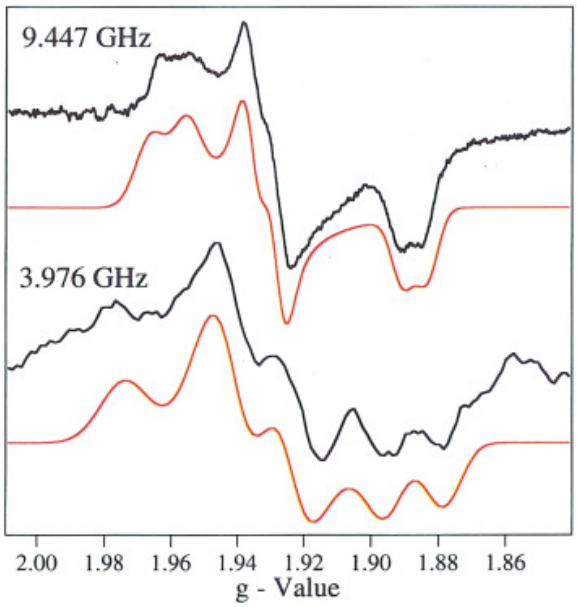


Figure 5. (a) X-band (9.447 GHz). (b) S-band (3.976 GHz) EPR spectra recorded under non-saturating conditions for W-DMSOR at 110 K after incubation with 2 mM sodium dithionite for four minutes, together with the corresponding simulated spectra: g_1, 1.9661; g_2, 1.9320; g_3, 1.8870; A_1, 1.28; A_2, 0.84; A_3, 0.94 mT. Blue represents the experimental data and red the simulation achieved. EPR spectra were measured at ~3.98 GHz and ~9.45 GHz on a Bruker ESP-300E and a Bruker EMX-Series spectrometer, respectively, of the UK EPSRC Continuous Wave EPR Service facility at The University of Manchester. Spectra were recorded for 0.2 mM samples of W-DMSOR maintained at 110 – 120 K, using 20 mW microwave power and 0.4 mT (4 G) modulation amplitude. Background spectra were recorded, aligned and subtracted as described previously (Bennett, *et al*., 1994) Integrations were carried out with respect to a Cu(II)-EDTA standard and included corrections for relative transition probabilities and temperature. The addition of 2 mM sodium dithionite to a solution of W-DMSOR produced an EPR signal in the g~2 region. After a prescribed incubation time at 23°C, each sample was frozen by plunging the EPR tube containing the sample into a solid CO_2/MeOH bath. The intensity of the W(V) signal increased as a function of incubation time up to four minutes, after which the signal diminished in intensity. The maximum concentration of W(V) produced was estimated as 15(±2) % by integrations of simulations of the experimental data. Computer simulations were carried out as described (Butler, *et al*., 1999); the simulations shown are the best attained at both frequencies using a single set of principal g-values, A-values and Euler angles.

The UV/visible spectra of Mo-DMSOR and W-DMSOR as isolated are shown in Figure 6. The spectra are distinctive: that of Mo-DMSOR is in good agreement with previous reports McAlpine et al 1998, McEwan et al 1991, Bastian et al 1991; that of W-DMSOR has a similar profile to its Mo counterpart with the λ_{max}-values blue-shifted by ca 150 nm (i.e. 3000–5000 cm^{-1}). The absorptions are considered to arise from ligand-to-metal charge-transfer transitions, from sulfur-based orbitals to a d^0 metal centre. Consistent with this view, the blue-shift observed is similar to that (ca 4350 cm^{-1} from Mo to W) for the two lowest energy transitions of the $[MS_4]^{2-}$ (M = Mo, W) anions (Diemann & Müller, 1973).

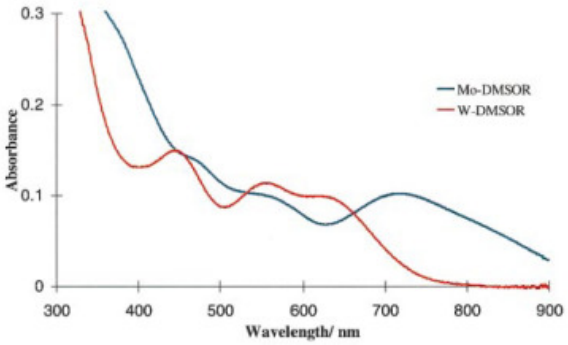


Figure 6. UV/visible absorption spectra, recorded at room temperature for: Mo-DMSOR (5 mg ml^{-1} in 50 mM Hepes, pH 7.5) (blue) and W-DMSOR (5 mg ml^{-1} in 50 mM Tris, pH 8.0) (red); spectra were recorded on a Perkin-Elmer Lambda 16 spectrophotometer.

The activity of W-DMSOR was measured, as described for Mo-DMSOR (Adams *et al*., 1999), using dithionite-reduced MV as the electron donor. The steady state rate of DMSO reduction is 27(±1) s^{-1} for Mo-DMSOR, but 470(±10) s^{-1} for W-DMSOR. In the DMS:PES/DCPIP oxidoreduction assay (Adams *et al*., 1999), the activity of Mo-DMSOR and W-DMSOR are 8.5(±0.1) s^{-1} and $\leqslant$0.05 s^{-1} molecules of DCPIP reduced (= DMS oxidised), respectively; the latter value could correspond to the presence of a small amount (ca 0.5 %) of Mo-DMSOR in the W-DMSOR. This difference in activity is attributed to the different redox potentials of the Mo and W centres of the DMSOR. EPR potentiometric titrations have been accomplished for both Mo and W-DMSOR (Hagedoorn *et al*., 2000). The results obtained for Mo-DMSOR are in good agreement with those of Bastian *et al.*(1991) who reported midpoint potentials (*versus* NHE) of +141 mV for the Mo(VI)/(V) couple and +200 mV for the Mo(V)/(IV) couple at pH 7.0 and 173 K; the values for W-DMSOR under the corresponding conditions are −203 mV for W(VI)/W(V) couple and −105 mV for the W(V)/(IV) couple. Thus, as compared with Mo-DMSOR, W-DMSOR favours adoption of the higher (+VI and +V) oxidation states by some 325 mV.

Conclusions

The characterisation of W-DMSOR, reported here, clearly demonstrates that tungsten can directly replace molybdenum at the catalytic centre of a molybdoenzyme and produce an active enzyme. As isolated, W-DMSOR is in the W(VI) oxidation state, and the enzyme is able to access the same range of oxidation states (VI, V, and IV) as Mo-DMSOR Baugh et al 1997, Bennett et al 1994. UV/visible and EPR spectra indicate that the electronic structures of the W(VI) and W(V) centres, respectively, are very similar to their molybdenum counterparts. The tungstoenzymes identified to date catalyse oxygen transfer at a carbon site Johnson et al 1996, Hagen and Arendson 1998; our studies show that reduction at a sulfur centre can be catalysed by a tungstoenzyme. W-DMSOR is significantly more active than Mo-DMSO for the reduction of DMSO but, in contrast to Mo-DMSOR, displays no discernible ability to catalyse the oxidation of DMS. These differences are consistent with W(IV) being a stronger reductant than Mo(IV) and (correspondingly) W(VI) being a weaker oxidant than Mo(VI). This difference in redox properties, together with the relative bio-availability of the metals, are suggested to be major factors in determining whether an organism uses molybdo- or tungstoenzymes.

Protein Data Bank accession number

The final coordinates of the structure determination for oxidised W-DMSOR have been deposited in the RCSB Protein Data Bank, reference number 1e18.

Acknowledgements

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