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Redox characteristics of the tungsten DMSO reductase of *Rhodobacter capsulatus*

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Abstract The dimethylsulfoxide reductase (DMSOR) from *Rhodobacter capsulatus* is known to retain its three-dimensional structure and enzymatic activity upon substitution of molybdenum, the metal that occurs naturally at the active site, by tungsten. The redox properties of tungsten-substituted DMSOR (W-DMSOR) have been investigated by a dye-mediated reductive titration with the concentration of the W^V state monitored by EPR spectroscopy. At pH 7.0, $E_m(W^{VI}/W^V)$ is −194 mV and $E_m(W^V/W^{IV})$ is −134 mV. Each E_m value of W-DMSOR is significantly lower (220 and 334 mV, respectively) than that of the corresponding couple of Mo-DMSOR. These redox potentials are consistent with the ability of Mo-DMSOR to catalyze both the reduction of DMSO to DMS and the back reaction, whereas W-DMSOR is very effective in catalyzing the forward reaction, but shows no ability to catalyze the oxidation of DMS to DMSO.

Key words: Dimethylsulfoxide reductase; Tungsten; Molybdenum; Midpoint potential; Electron paramagnetic resonance

1. Introduction

Molybdenum (Mo) enzymes occur in all living systems and, with the notable exception of the nitrogenases, involve the metal bound by one or two molecules of a special cofactor 'molybdopterin' (MPT) [1–3]. The Mo enzymes catalyze a conversion, the net transfer of an oxygen atom to or from the substrate. In each case, the conversion is effected at the Mo center and the catalytic cycle involves interconversion between Mo^{VI} and Mo^{IV} oxidation states. The electron paramagnetic resonance (EPR)-active Mo^V state is generated by a one-electron reduction of the Mo^{VI} state (or a one-electron oxidation of the Mo^{IV} state); this can occur during the catalytic cycle en route to the reformation of the catalytically active state, or by chemical/electrochemical reduction (or oxidation).

Tungsten (W) possesses similar chemical properties to Mo [4] and several W-containing enzymes have been isolated and characterized [2,3,5–7]. These enzymes involve W bound to two MPTs and the majority of these systems – like their Mo counterparts – catalyze a conversion, the net effect of which is the transfer of an oxygen atom to or from the substrate. Furthermore, it has been interesting to compare the properties of a Mo enzyme with those of its W-substituted analogue. For example, W can replace Mo in sulfite oxidase [8]. This substitution permitted the first direct comparison of the EPR spectrum of a Mo^V center of an MPT-dependent enzyme with that of its W^V counterpart; the characteristics of these spectra suggested that the two metals were bound at the same site. However, the properties of the two systems are not identical. Thus, the Mo center of sulfite oxidase is readily reduced from Mo^{VI} to Mo^V, in the pH range 6–9, but reduction of the W^{VI} center requires a $pH < 6$. These observations are consistent with reduction of the M^{VI} state involving coupled electron–proton addition [9] and a preference of $W > Mo$ for adoption of the M^{VI} state.

For sulfite oxidase, as with several other Mo MPT-dependent enzymes, substitution of the Mo by W leads to a loss of activity [10]. However, studies of *Rhodobacter capsulatus* dimethylsulfoxide reductase (DMSOR) [10–12] and *Escherichia coli* TMAOR (trimethylamine *N*-oxide reductase) [13] have shown that these enzymes retain some activity when W replaces Mo. Furthermore, X-ray crystallographic and X-ray absorption spectroscopic studies [11] have shown that *R. capsulatus* DMSOR incorporates W into the same site as Mo [14,15], without any significant changes in the structure of the protein and the nature and the dimensions of the binding site.

Here we report redox titrations accomplished for *R. capsulatus* W-DMSOR to determine the potentials of the W^{VI}/W^V and W^{V}/W^{IV} couples over the pH range 5.0–8.0. This information, when compared with the potentials of the Mo^{VI}/Mo^V and Mo^V/Mo^{IV} couples of the Mo-DMSOR of *R. capsulatus* [16] and *Rhodobacter sphaeroides* [17,18] provide valuable information to aid our understanding of the similarities and differences in behavior of Mo and W as the catalytic centers of MPT-dependent enzymes [9].

2. Materials en methods

2.1. Growth and protein purification

R. capsulatus was grown on medium containing tungstate and the periplasmic W-DMSOR was isolated and purified as previously de-

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scribed [11]. The W content was determined electroanalytically [19] and samples employed for the redox titrations had ca. 0.6 W/molecule.

2.2. Redox titrations

The oxidation/reduction characteristics of *R. capsulatus* W-DMSOR were investigated by a dye-mediated reductive titration, as described previously [20]. These studies were accomplished at 25°C in 100 mM potassium phosphate buffer, at pH 6.0, 7.0 and 8.0, and 100 mM citrate–phosphate buffer at pH 5.0; each medium contained 60 μM W-DMSOR. These buffers were used since HEPES buffer has been shown to bind at the active site of Mo-DMSOR of *R. sphaeroides* [15] and to induce dissociation of the dithiolene sulfurs of MPT from the metal [21]. Sodium dithionite was used as the reductant and potassium ferricyanide as the oxidant; samples were immediately frozen at 77 K upon attainment of redox equilibrium.

2.3. Spectroscopy

X-band EPR spectra were recorded on a Bruker ER-200D spectrometer, using facilities and data handling as detailed elsewhere [22]. The W^V EPR signals observed during the course of the redox titrations were simulated as described previously [23].

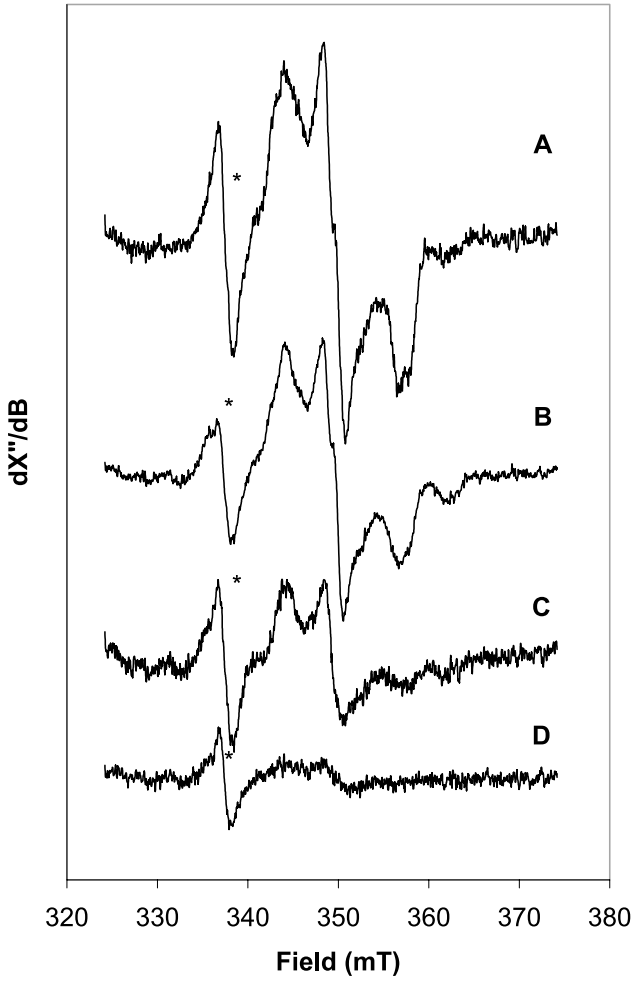

Fig. 1. EPR spectra observed for W-DMSOR over the pH range 5.0–8.0. W-DMSOR in 100 mM phosphate–citrate buffer, pH 5.0 (A); 100 mM KPi buffer, pH 6.0 (B), 7.0 (C) or 8.0 (D). Sodium dithionite was used as the reductant. All spectra have been normalized with respect to the tungsten concentration of the sample. The signal labeled * is attributed to radicals that derive from the redox mediators methyl viologen and benzyl viologen, used in the titration. EPR conditions: microwave frequency, 9.43 GHz; modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; microwave power, 5.0 mW; temperature, 44 K.

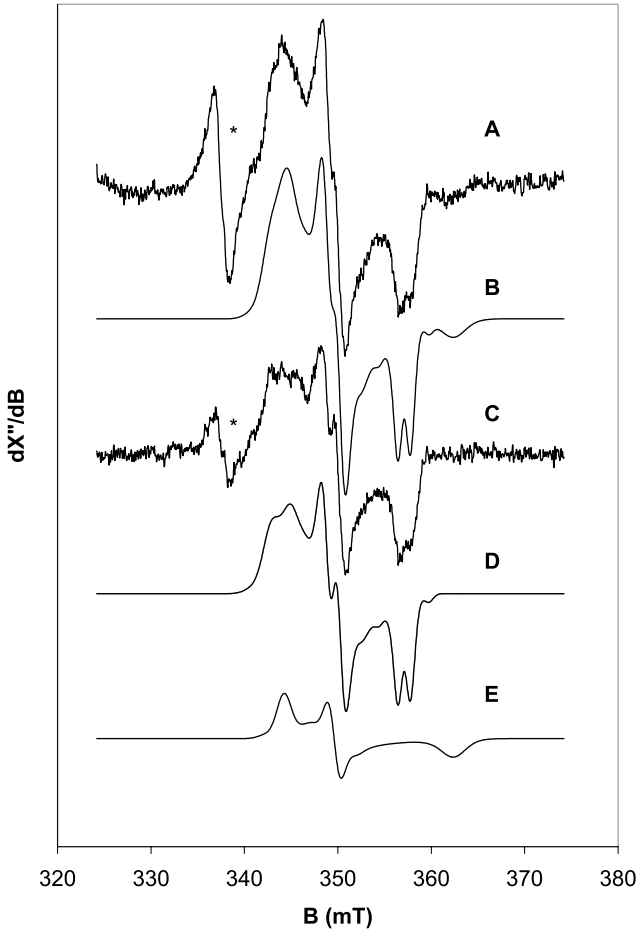

Fig. 2. EPR spectra observed for W-DMSOR together with simulations of the 'split' and 'unsplit' signals. A: Spectrum observed at pH 5.0. B: Simulation produced by a combination of simulated spectra, 40% 'split' and 60% 'unsplit'. C: Spectrum at pH 5.0 minus that at pH 7.0 revealing the 'split' signal. D: Simulation of the 'split' signal. E: Simulation of the 'unsplit' signal. The signal labeled * is attributed to radicals that derive from the redox mediators methyl viologen and benzyl viologen, used in the titration. EPR conditions are the same as in the legend to Fig. 1. Simulation parameters: 'split' signal, $g_{xyz} = 1.888$, 1.927 and 1.960, $A(^{183}W)_{xyz} = 4.0$, 4.0 and 4.0 mT, $A(^{1}H)_{xyz} = 1.3$, 1.4 and 2.0 mT, linewidth $W_{xyz} = 0.8$, 0.83 and 1.5 mT; 'unsplit' signal, $g_{xyz} = 1.860$, 1.928 and 1.958, $A(^{183}W)_{xyz} = 4.0$, 4.0 and 4.0 mT, $W_{xyz} = 2.0$, 1.0 and 1.3 mT.

3. Results

3.1. W^V EPR signals observed for W-DMSOR

Both the shape and the intensity of the EPR spectrum of the W^V species observed at potentials from −250 −50 mV were found to be pH dependent (Fig. 1). The EPR spectra have been interpreted (Fig. 2) on the premise of the presence of two different W^V species; one signal manifests a clear superhyperfine coupling, but the other does not. These signals are designated as 'split' ($g_{xyz} = 1.888$, 1.927, 1.960) and 'unsplit' ($g_{xyz} = 1.860$, 1.928, 1.958), respectively. The former is considered to arise from a W^V ($5d^1$) center, in which the unpaired electron is coupled to a proton; consistent with this view, the 'split' signal is predominant at pH 5.0 and the 'unsplit' signal is predominant at pH 7.0. The 'split' spectrum (Fig. 2C), obtained by subtraction of the spectrum at pH 7.0 from that at pH 5.0 (the spectrum at pH 7.0 is normalized

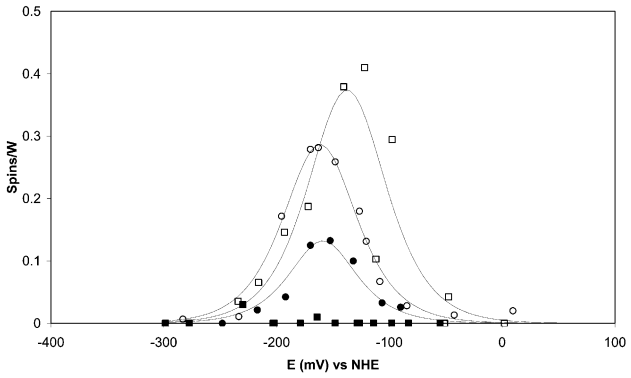

Fig. 3. Amount of W^V (normalized with respect to the total amount of tungsten present in the sample) present in W-DMSOR as a function of the redox potential of the medium at pH 5.0 (□), 6.0 (○), 7.0 (●) and 8.0 (■). The experimental data for each pH have been fitted (solid line) to the Nernst equation, on the basis of two one-electron couples, W^{VI}/W^V and W^V/W^{IV}, with the midpoint potentials given in Table 1.

in such a way that the g_x of the 'unsplit' signal in the spectrum at pH 5.0 disappears after subtraction), closely resembles the W^V EPR spectrum previously reported for dithionite-reduced *R. capsulatus* W-DMSOR [11].

3.2. $E_m(W^{VI/V})$ and $E_m(W^{V/IV})$ of W-DMSOR

The redox titration curves obtained for W-DMSOR at pH 5.0, 6.0, 7.0 and 8.0 are shown in Fig. 3. The maximum quantity of W^V, ca. 40% of the W present in the sample, occurs at pH 5.0. The redox titration curves were interpreted on the basis of two redox couples: a pH-dependent $E_m(W^{VI/V})$ couple and a pH-independent $E_m(W^{V/IV})$ couple; the midpoint potentials of these couples are given in Table 1. The pH dependence of the $E_m(W^{VI/V})$ is −31 mV/pH unit, a value significantly lower than the −59 mV/pH unit that is expected for a one-electron reduction coupled to the uptake of one proton.

The values of $E_m(Mo^{VI/V})$ and $E_m(Mo^{V/IV})$ for Mo-DMSOR of *R. sphaeroides*, as measured by redox titrations, have been reported as +141 and +200 mV (pH 7.0) [18] and +37 and +83 mV (pH 8.5) [17]. The values of $E_m(Mo^{VI/V})$ and $E_m(Mo^{V/IV})$ for *R. capsulatus* Mo-DMSOR have only been as measured by direct electrochemistry of the enzyme adsorbed at a pyrolytic graphite electrode in the presence of surfactants [16]. Therefore we performed a control titration with *R. capsulatus* Mo-DMSOR at pH 8.0, which produced values for $E_m(Mo^{VI}/Mo^V)$ and $E_m(Mo^V/Mo^{IV})$ of +26 and +200 mV, respectively. These values are reasonably consistent with the E_m values reported for *R. sphaeroides* [17,18] and the $E_m(Mo^{VI}/Mo^V) = +161$ mV obtained for *R. capsulatus* by the electrochemical investigation [16]. However, the $E_m(Mo^V/Mo^{IV})$ value obtained in the latter study was −102 mV. This value differs significantly from the $E_m(Mo^V/Mo^{IV})$ values obtained in the two redox titrations; thus, it is possible that the electrochemical investigation of this couple may represent the enzyme in a conformation different from that in the redox titrations.

Table 1
Midpoint potentials of the tungsten center in DMSOR

pH	$E_m(W^{VI}/W^V)$ (mV)	$E_m(W^V/W^{IV})$ (mV)
5.0	−133 ± 14	−142 ± 16
6.0	−167 ± 4	−156 ± 4
7.0	−194 ± 6	−134 ± 6
8.0	n.d.	n.d.

n.d. not determinable. The confidence limits have been set as two times the standard deviation of the fit to the E_m values.

4. Discussion

4.1. EPR properties of W-DMSOR

The 'split' W^V signal of W-DMSOR has $g_{av} = 1.925$, and the 'unsplit' W^V signal has $g_{av} = 1.916$. In the oxidized state of W-DMSOR, the metal is bound to an oxo-group, one serine, and four dithiolene sulfurs [11]. We propose that the 'split' signal is generated from the W^{VI} center by the coupled addition of one electron and one proton, to produce a $\{W^V(OH)(O_{Ser})(S_{dithiolene})_4\}$ center (Fig. 4). The 'unsplit' W^V EPR signal is considered to arise from the deprotonated form of this center, i.e. $\{W^V(O)(O_{Ser})(S_{dithiolene})_4\}$ (Fig. 4).

Several Mo^V EPR signals have been reported for reduced forms of Mo-DMSOR, however, only the 'high-*g*-split' and 'high-*g*-unsplit' signals are considered to be biochemically relevant; each signal has $g_{av} = 1.98$ [24,25]. The former has been attributed to a Mo^V center possessing an Mo^V-OH moiety and the latter to a center lacking such a group [25]. Based on the EPR spectroscopy we cannot discriminate between $\{W^V(O)(O_{Ser})(S_{dithiolene})_4\}$ or its deoxo form $\{W^V(O_{Ser})(S_{dithiolene})_4\}$ represented by the 'unsplit' W^V EPR signal.

A Mo^V center generally possesses a g_{av} value closer to the free-electron value than the corresponding W^V center, since the spin–orbit coupling constant of W^V ($\zeta = 2700$ cm^{-1}) is greater than that of Mo^V ($\zeta = 900$ cm^{-1}) [26,27]. The respective anisotropy ($g_z - g_x$) and rhombicity ($g_z - g_y / g_z - g_x$) of the 'split' and 'unsplit' W^V signals (and their Mo^V counterparts [18,24]) are: 0.071 (0.027) and 0.44 (0.41) and 0.096 (0.025) and 0.31 (0.25). Thus, for the M^V sites of *R. capsulatus* DMSOR, the anisotropy of each W^V signal is significantly greater than that of the corresponding Mo^V signal, a situation comparable to the situation for the Mo^V and W^V centers of sulfite oxidase [8] and $[M^VO(dithiolene)_2]^{1-}$ complexes [28,29].

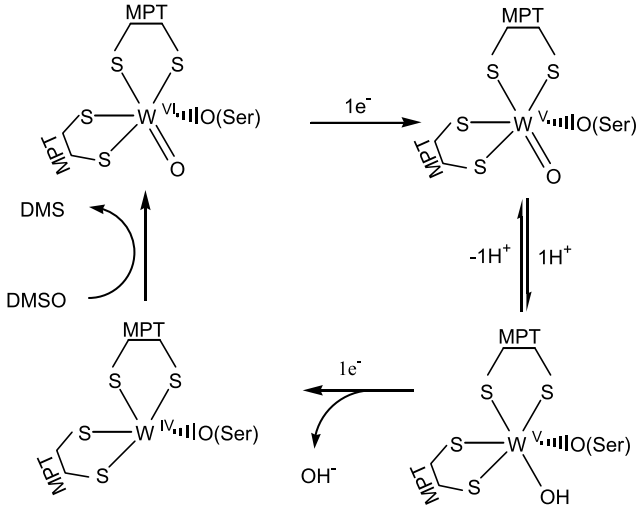

Fig. 4. Schematic view of a possible catalytic mechanism for the reduction of DMSO by W-DMSOR.

This difference is also attributed to the greater spin–orbit coupling constant of W^V vs. Mo^V.

4.2. Redox properties of W-DMSOR

The $E_m(W^{VI}/W^V)$ and $E_m(W^V/W^{IV})$ of *R. capsulatus* W-DMSOR have been determined by redox titrations monitored by EPR spectroscopy. The potentials were obtained by fitting the amount of W^V (expressed as a percentage of the total amount of W present) to the Nernst equation for two, sequential, one-electron processes. The determination of $E_m(M^{VI/V})$ and $E_m(M^{V/IV})$ values by this procedure for Mo and W enzymes is not trivial. The EPR detectable M^V species is usually present as an intermediate of the catalytic cycle that operates between M^{VI} and M^{IV} and usually comprises only a small percentage of the total metal content. Also, more than one M^V species may be observed. Thus, an accurate assessment of the amount of the relevant M^V present can be difficult to achieve, since the fit of the data to the Nernst equation for sequential $M^{VI} \rightarrow M^V \rightarrow M^{IV}$ processes is very sensitive to the amount of the M^V state. The uncertainty associated with the values obtained in the studies reported herein is estimated to be ca. ± 30 mV.

The intensity of the W^V EPR signal was observed to be a maximum at pH 5.0, decreasing with higher pH, consistent with a pH dependence of $E_m(W^{VI}/W^V)$ and/or $E_m(W^V/W^{IV})$. The values obtained for the midpoint potentials of these couples at pH 5.0, 6.0 and 7.0 are given in Table 1. The values at pH 7.0, $E_m(W^{VI}/W^V) = -194$ mV and $E_m(W^V/W^{IV}) = -134$ mV, differ slightly from those quoted in an earlier report [11]. This difference arises from the inclusion of an independent measurement of the total W content of the enzyme in the present, but not in the previous, study. The potential of the W^{VI}/W^V couple varies with pH to the extent of ca. -31 mV/pH unit.

The crystal structure of oxidized *R. capsulatus* W-DMSOR [11] has shown that the W^{VI} center is coordinated by the four dithiolene sulfurs from the two MPT ligands, one oxygen from Ser147 and one oxo-group. The environment of the W^{VI} is essentially the same as that of the six-coordinate Mo^{VI} center of oxidized *R. sphaeroides* Mo-DMSOR that is considered to be the catalytically competent site [15]. Given the structural congruency of the Mo and W sites of DMSOR, differences in the redox properties of these centers are attributable to the nature of the metal, as modulated by the ligands – notably the two MPTs [2]. In coordination chemistry, the potential at which a W^{VI} or W^V center is lower than its Mo analogue by 1000 to 225 mV depends on the nature of the ligands bound; at the lower end of this range are the $[MO(dithiolene)_2]^{1-}/[MO(dithiolene)_2]^{2-}$ couples, for which the value for M = W is ca. 225 mV less than that of the analogous couple with M = Mo [28–30]. The redox potential data obtained for *R. capsulatus* W-DMSOR is relevant to the significantly different behavior of this enzyme and that of its Mo counterpart [11]. At pH 8.0, the $E_m(Mo^{VI}/Mo^V)$ (+26 mV) and $E_m(Mo^V/Mo^{IV})$ (+200 mV) are close to the midpoint potential of the DMSO/DMS couple, +160 mV [31]; thus, Mo-DMSOR can catalyze both the reduction of DMSO to DMS and the back reaction. However, at pH 7.0, $E_m(W^{VI}/W^V)$ is -194 mV and $E_m(W^V/W^{IV})$ is -134 mV, which is 354 and 294 mV, respectively, lower than the midpoint potential of the DMSO/DMS couple, consistent with W-DMSOR being capable of catalyzing the reduction of DMSO (ca. 17 times faster than Mo-DMSOR), but showing no ability to catalyze the oxidation of DMS to DMSO. Differences in the redox potentials of Mo and W centers in enzymes, such as those reported herein, may – at least in part – explain why certain organisms (e.g. *Pyrococcus furiosus* [32]) employ W enzymes in preference to the more usual Mo systems.

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