The critical role of tryptophan-116 in the catalytic cycle of dimethylsulfoxide reductase from *Rhodobacter capsulatus*

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Abstract In dimethylsulfoxide reductase of *Rhodobacter capsulatus* tryptophan-116 forms a hydrogen bond with a single oxo ligand bound to the molybdenum ion. Mutation of this residue to phenylalanine affected the UV/visible spectrum of the purified Mo^{VI} form of dimethylsulfoxide reductase resulting in the loss of the characteristic transition at 720 nm. Results of steady-state kinetic analysis and electrochemical studies suggest that tryptophan 116 plays a critical role in stabilizing the hexacoordinate monooxo Mo^{VI} form of the enzyme and prevents the formation of a dioxo pentacoordinate Mo^{VI} species, generated as a consequence of the dissociation of one of the dithiolene ligands of the molybdopterin cofactor from the Mo ion.

Key words: Dimethylsulfoxide reductase; Molybdenum enzyme; Site-directed mutagenesis; Electrochemistry; *Rhodobacter capsulatus*

1. Introduction

The dimethylsulfoxide (DMSO) reductase family of molybdenum enzymes is one of four families of enzyme that contain a form of pterin molybdenum cofactor (Moco) [1]. In all enzymes of this superfamily a molybdenum atom coordinated by one or two *ene*-dithiolate ligands is provided by an organic component known as molybdopterin (MPT). A distinctive feature of the DMSO reductase family of MPT-containing enzymes is that the Mo is bound by four thiolate ligands provided by molybdopterin guanine dinucleotide (MGD) moieties [1,2]. Enzymes of the DMSO reductase family are thus far limited to prokaryotes where they play an important role in anaerobic respiration and lithotrophy [3].

The X-ray crystal structure of several members of the DMSO reductase family has been determined. These include periplasmic DMSO reductase [4–8], periplasmic nitrate reductase (NAP) [9], formate dehydrogenases (FDH-H and FDH-N) [10,11], trimethylamine-*N*-oxide (TMAO) reductase [12] and arsenite oxidase (ASO) [13]. The common protein structural features of these enzymes give no indication as to how their specific catalytic properties are determined. However, an examination of the Mo active site does reveal some significant differences between enzymes of the DMSO reductase family. In DMSO reductase and TMAO reductase a fifth ligand to the Mo is provided by a serine side chain [4,12], while in NAP it is a cysteine residue [9] and in FDH a selenocysteine residue [10]. In ASO, there is no amino acid ligand to the Mo atom [13]. In addition, a terminal oxygen ligand is coordinated to the Mo ion (oxo, hydroxo or aqua) [4,6,9,10,12,13]. Since almost all Moco-containing enzymes catalyze an oxygen atom transfer it seems likely that differences in the Mo active sites represent the tuning of their thermodynamic and structural properties to allow distinct substrates to be used.

A further level of diversity in the DMSO reductase family can be seen in the interaction between the Mo active site and nearby amino acid residues which are not directly coordinated to the Mo center. In the crystal structure of DMSO reductase it has been observed that a tryptophan (residue 116) hydrogen-bonds to an oxo group coordinated to the Mo ion [5,6,8]. This tryptophan residue is conserved in TMAO reductase, which is almost structurally identical to DMSO reductase [12]. In DMSO reductase another amino acid side chain provided by Y114 is also located close to the Mo active site [8]. Although recent studies have indicated that this residue does not H-bond to the Mo-oxo group [8] two recent studies have shown that Y114 plays a role in defining the substrate specificity and kinetic parameters of DMSO reductase [14,15]. In the present paper we report the characterization of a W116→F mutant DMSO reductase from *Rhodobacter capsulatus*. This enabled us to investigate the effect of the removal of the hydrogen bonding interaction between the Nε1 hydrogen of W116 and the Mo-oxo group.

2. Materials and methods

2.1. Mutagenesis and expression of DMSO reductase

The mutagenesis of the *dorA* gene encoding DMSO reductase (accession number U49506) in *Rb. capsulatus* was carried out essentially as described in [15]. Briefly, complementary primers were designed against the *dorA* nucleotide sequence containing the appropriate changes to introduce the W116F mutation (numbering follows [5], accession no. 1DMS). The primers used were W116Ffwd, 5′-GGC-

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Abbreviations: MPT, molybdopterin; DMSO, dimethylsulfoxide; MGD, molybdopterin guanine dinucleotide; NAP, periplasmic nitrate reductase; FDH, formate dehydrogenase; ASO, arsenite oxidase; TMAO, trimethylamine-*N*-oxide; MV, methyl viologen; DMS, dimethylsulfide; DCPIP, dichlorophenol indophenol; DDAB, didodecyldimethylammonium bromide; CSIRO, Commonwealth Scientific and Industrial Research Organization; ICP-MS, inductively coupled plasma mass spectrometry

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TCCTATGGCTTCAAAAGCCCCGGG-3′, and W116Frev, 5′-CCCGGGGCTTTTGAAGCCATAGGAGCC-3′. These primers were then used to mutate the construct, pUCJRDor [15], using the Quikchange XL site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol. The entire fragment of the *dor* operon contained in pUCJRDor was then sequenced to ensure that no non-specific mutations had occurred. This fragment was then cloned into pJP5603 to form the construct pJP5603W116F. This construct was then conjugated into the *Rb. capsulatus* strain 37b4Δ*dorA* as described previously [15]. The expression of mutant DMSO reductase was confirmed by Western blotting. The genomic DNA of clones expressing DMSO reductase was screened as described previously to confirm the presence of the mutation [15]. One clone was then selected for further study and named 37b4W116F.

2.2. Purification of W116F DMSO reductase

Strain 37b4W116F was grown under phototrophic condition in RCV medium supplemented with the appropriate antibiotics until the culture reached late log/early stationary phase. DMSO reductase production was then induced with the addition of 15 mM DMSO and growth for a further 12 h. DMSO reductase was then purified from 37b4W116F as described previously [15]. Purity of the sample was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis.

2.3. UV/visible spectroscopy and steady-state kinetic analysis

All UV/visible spectroscopy was carried out using a Hitachi U-3000 spectrophotometer. Steady-state kinetics of dithionite-reduced methylviologen (MV):DMSO oxidoreductase activity and PES-dependent dimethylsulfide (DMS):dichlorophenol indophenol (DCPIP) oxidoreductase activity were measured as described previously [16,17]. Determination of k_{cat} and K_m values were carried out in triplicate. Values of k_{cat} are expressed as moles of DCPIP or reduced MV consumed per mole of enzyme per second giving units of s^{-1}.

2.4. Electrochemistry and electrode preparation

Electrochemical measurements were performed with a BAS100B/W workstation employing a conventional three electrode system comprising an edge plane pyrolytic graphite working, Pt wire counter and Ag/AgCl reference electrodes. Experiments were performed at 25°C within a Belle Technology glovebox under an atmosphere of N_2 (O_2 concentration less than 2 ppm). The electrochemical cell volume was approximately 500 μl, and the solution pH was varied from 5 to 10 using the buffer mixture of Bis-Tris propane (10 mM) and 2-amino-2-methylpropan-1-ol (10 mM), with 0.01 M NaCl as supporting electrolyte.

A clean working electrode surface was obtained by cleaving a ca. 1 μm layer from the face of the electrode using a microtome followed by sonication in distilled water. No abrasives were used. A surfactant film of the enzyme was prepared and adsorbed to the surface of the electrode as described previously [18]. A solution of protein (10 μl, 86 μM) was combined with a 15 μl solution comprising didodecyldimethylammonium bromide (DDAB, 2 mM) and Tris buffer (50 μM). The working electrode surface was coated with this mixture and then allowed to dry for a few hours at 6°C.

2.5. Determination of ε_{280} and Mo content

For native DMSO reductase accurate determination of the concentration of a solution of purified enzyme relied on the previously published extinction coefficient for the long-wavelength transition (ε_{725} = 2 mM^{-1} cm^{-1}). However, as described below the W116F mutant DMSO reductase lacked this transition and therefore this could not be used to determining the concentration of a solution of this form of the enzyme. It was essential to determine accurately the concentration of W116F DMSO reductase to allow the determination of accurate kinetic data and Mo:DMSO reductase molar ratio. To achieve this total amino acid analysis of a solution of W116F DMSO reductase with a known OD_{280} was carried out. The resulting molar amount of each amino acid in the sample was then used to calculate an extinction coefficient for W116F DMSO reductase of ε_{280} = 223 mM^{-1} cm^{-1}. This coefficient was used in all subsequent experiments to determine the concentration of W116F DMSO reductase present. The Mo content of duplicate samples of the wild-type and W116F DMSO reductase was determined using inductively coupled plasma mass spectrometry (ICP-MS) at CSIRO Livestock Industry, Indooroopilly, Queensland.

3. Results

3.1. UV/visible spectroscopy

Fig. 1 shows a comparison of the UV/visible absorption spectra of the resting Mo^{VI} forms of native and W116F DMSO reductase between 300 nm and 800 nm. The most striking feature of the spectrum of the W116F enzyme was the absence of the long-wavelength transition which in the native enzyme is centered at 720 nm. The two transitions present in the spectrum of the native enzyme at 480 and 550 nm were still present in the spectrum of W116F DMSO reductase (Fig. 1) but their intensity was increased. The electronic transition centered at 380 nm was more defined in the spectrum of the W116F DMSO reductase compared to the native enzyme due in part to the deepening of the trough centered at 350 nm in the mutant enzyme. Addition of DMS to native DMSO reductase causes the formation of a pink species with a characteristic spectrum [7]. This has been shown to be a Mo^{IV} complex with DMSO bound [19]. Bray and co-workers have also shown that addition of DMSO to the resting native enzyme also causes a change in the spectrum [20]. However, the addition of DMSO or DMS at concentrations of up to 28 mM and 25 mM respectively caused no appreciable changes in the spectrum of the W116F form of DMSO reductase.

3.2. Mo content of W116F DMSO reductase

Observation of the loss of the long-wavelength transition in the optical spectrum for the W116F DMSO reductase immediately led to concern over the integrity of the active site and whether the Mo atom had been lost. This seemed unlikely because of the presence of absorption bands in the visible region which almost certainly arise from ligand-to-metal charge transfer. Native and W116F DMSO reductase samples of known concentration were analyzed for Mo content. The ratio of Mo/polypeptide in the W116F form of DMSO reductase was determined to be 0.66 while the ratio seen in the native DMSO reductase was 0.57 (Table 1). These data show that spectral differences between the two forms of DMSO reductase do not arise from a difference in Mo content.

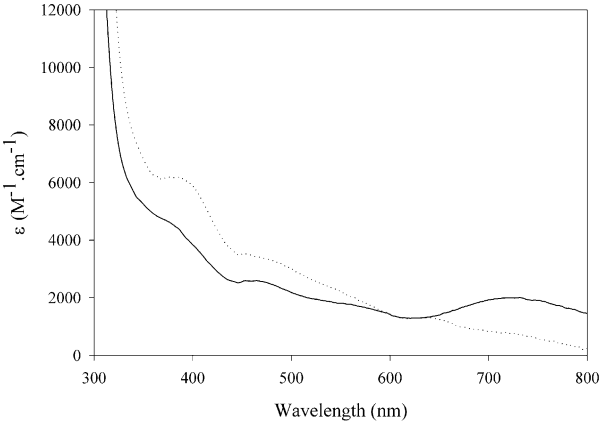

Fig. 1. UV/visible absorption spectra of 'as prepared' oxidized form of native DMSO reductase (solid line) and purified W116F mutant DMSO reductase (dotted line).

Table 1
Molybdenum content and steady-state kinetic parameters determined for the native and W116F DMSO reductase from *Rb. capsulatus* for the substrates DMSO and TMAO

	Mo content[a]	MV:DMSO oxidoreductase			MV:TMAO oxidoreductase		
	(mole mole^{-1})	k_{cat} (s^{-1})[b]	K_m (μM)[c]	k_{cat}/K_m (μM^{-1} s^{-1})	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
Native	0.57	42.9 ± 0.6	9.7 ± 1.2	4.4 ± 0.7	134.5 ± 4.9	193.8 ± 9.8	0.7 ± 0.1
W116F	0.66	7.0 ± 0.2	26.1 ± 3.1	0.3 ± 0.0[d]	11.3 ± 0.3	95.9 ± 11.0	0.1 ± 0.0[e]

[a]Mo content was determined by ICP-MS.
[b]k_{cat} values are given to one decimal place and are the mean of three determinations in each case (± S.E.M.).
[c]K_m values are given to one decimal place and are derived from a non-linear fit of a plot of the average of three determinations at each substrate concentration of [substrate] (mM) vs. velocity (s^{-1}) (± S.E.M.).
[d]Actual value 0.03.
[e]Actual value 0.02.

3.3. Steady-state kinetic analysis

DMSO reductase activity was assayed using dithionite-reduced MV as electron donor. The results in Table 1 show that the W116F form of DMSO reductase had a low k_{cat} towards both DMSO and TMAO, retaining approximately 17% and 8% respectively of the activity seen in the wild-type DMSO reductase. The K_m for DMSO in the W116F mutant DMSO reductase was about three times higher than that seen for the native enzyme. Interestingly, the K_m of the W116F mutant enzyme for TMAO was approximately half the value seen for the native DMSO reductase. For both substrates the value of k_{cat}/K_m was reduced for the W116F mutant when compared to the native enzyme. It has been demonstrated that reduced MV not only acts as an electron donor to DMSO reductase but is also able to activate inactive forms of the enzyme which differ in the structure of the Mo active site compared to the native enzyme [20,21]. Thus, the use of dithionite-reduced MV can be misleading with respect to the understanding of the relationship between active site structure and catalysis. It has been suggested that the PES-dependent DMS:DCPIP oxidoreductase activity of DMSO reductase is a more accurate measure of a catalytically active form of the enzyme [20]. The W116F form of DMSO reductase had no measurable activity in this assay with either DMS or trimethylamine as substrates. Furthermore, turnover of the mutant enzyme with dithionite-reduced MV and DMSO did not generate an enzyme with PES-dependent DMS:DCPIP oxidoreductase activity.

3.4. Electrochemical measurements

Cyclic voltammetry and square wave voltammetry were employed to determine the midpoint potentials of the Mo center as well as their pH dependence. Well separated reversible $Mo^{VI/V}$ and $Mo^{V/IV}$ responses were observed with the enzyme immobilized within a DDAB surfactant film adsorbed onto an edge plane pyrolytic graphite working electrode (Fig. 2a). The apparent midpoint potentials for the W116F mutant have been plotted in Fig. 2b (circles) as a function of pH, and the corresponding potentials reported for the native enzyme under identical conditions [18] are included for comparison (triangles). In the W116F mutant, both the higher potential $Mo^{VI/V}$ couple and the $Mo^{V/IV}$ couple were observed to be pH-dependent, and the slope of each profile is approximately −59 mV/pH unit. The $Mo^{VI/V}$ couple of the native enzyme exhibits a slight cathodic shift but shows similar pH dependence. However, the $Mo^{V/IV}$ couple of the native enzyme is pH-independent, indicating a crucial role for the W116/F116 residues in the native and mutant enzymes during electron transfer.

4. Discussion

It is now accepted that the active form DMSO reductase is hexacoordinate with ligands provided by four thiolates from the two MGD groups, a hydroxymethyl ligand from S147 and a single oxo group [1,3]. This oxo group is equivalent to oxo-2

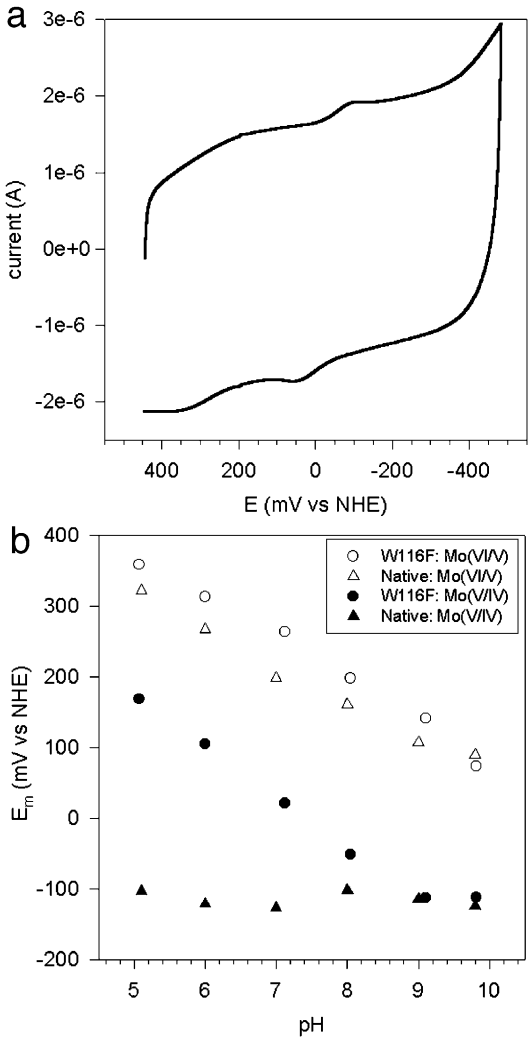

Fig. 2. a: Cyclic voltammogram of the W116F mutant of DMSO reductase at DDAB surfactant-modified edge plane pyrolytic graphite working electrode. Scan rate is 50 mV/s. b: Plot of midpoint redox potentials of the Mo center of DMSO reductase vs. pH. $Mo^{VI/V}$ couple, native enzyme, open triangles; W116F, open circles; $Mo^{V/IV}$ couple, native enzyme, closed triangles; W116F, closed circles.

identified by Bailey and co-workers as the oxo group that is reactive towards DMS [7]. This oxo group is H-bonded to W116 and so it might have been predicted that disruption of this H-bonding would affect catalysis. The results presented herein showed that a W116F mutation caused a reduction in k_{cat} towards DMSO and TMAO. In contrast, the K_m for DMSO in the W116F mutant is not markedly affected (three-fold increase) and the K_m for TMAO is in fact reduced. These data would imply that W116 has an important role in the catalytic cycle of DMSO reductase but does not greatly influence the binding of the substrate. Recently, the equivalent tryptophan residue in the closely related biotin sulfoxide reductase (BSO) was mutated and analyzed [22]. A W90F mutant of BSO also exhibited a much reduced k_{cat} value towards a range of substrates compared to native BSO but K_m was not greatly affected.

Site-directed mutagenesis studies have demonstrated that Y114 in DMSO reductase has a critical role in catalysis [14,15]. It is notable that Y114 is absent from TMAO reductase [12], consistent with the view that this amino acid plays a critical role in S–O bond breakage during catalysis but is not essential for N–O bond cleavage. A Y114F mutation caused small and subtle changes to the UV/visible spectrum of the resting Mo^{VI} form of DMSO reductase [15]. As shown above the effect of a W116F mutation is much more dramatic. The transitions seen in the visible region of the spectrum of the DMSO reductase arise from ligand-to-metal charge transfer. Therefore, these absorption bands will be highly sensitive to a change in ligating atom as well as any changes to the symmetry of the Mo site.

The plasticity of the Mo active site has been the subject of extensive discussion [8,23,24]. Schindelin and co-workers have observed that there is discrete disorder at the Mo active site in the 1.3 Å crystal structure of DMSO reductase from *Rb. sphaeroides* [8]. The structure may be interpreted as a superposition of two distinct active sites: a hexacoordinate mono-oxo structure and a pentacoordinate dioxo structure in which one dithiolene group (from the Q-MGD) is outside the Mo coordination sphere [8]. The crystal structures of a number of inactive forms of DMSO reductase have also been described [23,24]. One of these forms, known as $DMSOR_{mod}H$, is formed by treatment of resting DMSO reductase with HEPES buffer. This results in movement of the Mo such that the thiolates of the Q-MGD are no longer within the Mo coordination sphere and results in the formation of a pentacoordinate dioxo Mo site [23]. HEPES treatment resulted in bleaching of the 720 nm absorption band in DMSO reductase and a loss of other visible absorption bands in the visible region. Based on a comparison of the spectrum of the W116F mutant form of DMSO reductase with that of $DMSOR_{mod}H$ we suggest that the W116F mutant enzyme also has a pentacoordinate dioxo Mo center. Another similarity between $DMSOR_{mod}H$ and the W116F mutant is low activity in the back assay with DMS as electron donor. Indeed, the W116F mutant has no PES-dependent DMS:DCPIP oxidoreductase activity and is unable to form a complex with DMS. 'Redox-cycling' of $DMSOR_{mod}H$ with dithionite-reduced MV and DMSO regenerated an active form of the enzyme which can subsequently catalyze the back reaction with DMS as electron donor [23]. In contrast, redox cycling of the W116F mutant does not restore DMS:DCPIP oxidoreductase activity.

On the basis of the above observations we suggest that presence of the side chain of Y114, a requirement for an S-oxide reductase with a high affinity for sulfoxides, creates

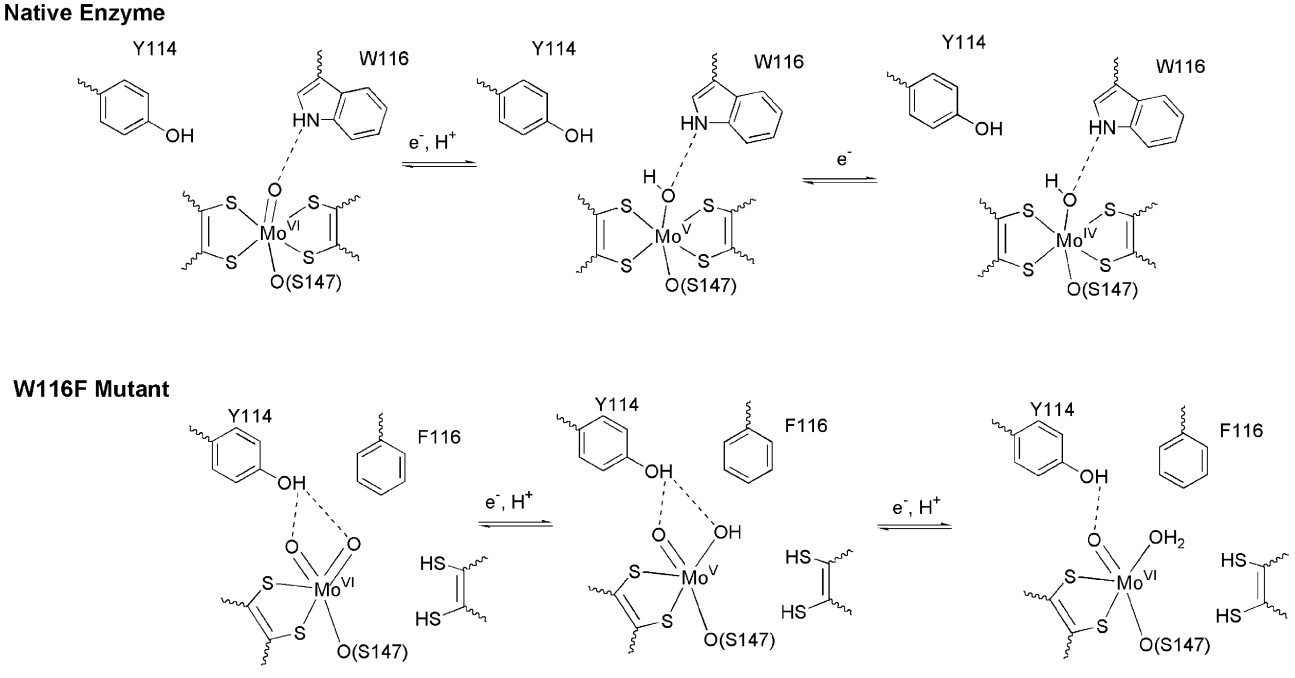

Fig. 3. Scheme showing proposed protonation reactions associated with electron transfer in native DMSO reductase and the W116F mutant enzyme.

instability in the Mo active site of DMSO reductase since this side chain can in principle also H-bond to a Mo-oxo group. We suggest that the critical role of W116 in DMSO reductase is to H-bond to this Mo-oxo group and prevent movement of the Mo-oxo unit away from the coordinating dithiolene of the Q-MGD in the resting Mo^{VI} enzyme. When no H-bonding amino acid side chain is present (as in the W116F mutant) then the enzyme is stabilized in the inactive pentacoordinate dioxo form.

W116 also influences the reductive steps in the conversion of Mo^{VI} to Mo^{IV}. A comparison of the electrochemistry of native DMSO reductase [18] and the W116F mutant reveals that in both enzymes, the $Mo^{VI/V}$ couple exhibits a pH dependence of −59 mV/pH unit, which is indicative of a single electron/single proton transfer reaction. We have previously assigned the site of protonation as the oxo ligand adjacent to W116 [18]. The influence of the W116 residue on the $Mo^{V/IV}$ couple is profound. In native DMSO reductase, the $Mo^{V/IV}$ couple is pH-independent in the range $5 < pH < 10$, which is consistent with reduction of the hydroxo-Mo^{V} form to the hydroxo-Mo^{IV} analogue with no coupled proton transfer. In the W116F mutant, a −59 mV/pH unit shift in the $Mo^{V/IV}$ is seen in the range $5 < pH < 9$, which may be assigned to a second single electron/single proton transfer reaction resulting in a fully reduced aqua complex.

These results may be rationalized by reference to the scheme shown in Fig. 3. The pH independence of the $Mo^{V/IV}$ couple in native DMSO reductase suggests that W116 remains H-bonded to the hydroxo ligand during both electron transfer steps. The hydroxo ligand, whilst accepting an H-bond from W116, is unable to concurrently accept a proton upon reduction to the tetravalent state. In the W116F mutant, the phenyl ring is unable to participate in H-bonding with the oxo/hydroxo-molybdenum center. The absence of an H-bonding interaction involving the terminal oxygen ligand must enhance its basicity in all oxidation states relative to the native enzyme. The small anodic shift in the $Mo^{VI/V}$ couple seen for the W116F mutant indicates that the pentavalent state is being stabilized, or that the hexavalent state is destabilized relative to the native enzyme. The latter hypothesis is more likely given that the oxo-Mo^{VI} moiety in the W116F mutant lacks the stabilizing influence of the W116 H-bond donor whereas the Mo^{V} moieties are each able to be stabilized by protonation, as opposed to H-bond formation, in the native and mutant enzymes. The Mo(V) electron paramagnetic resonance (EPR) spectrum of molybdenum enzymes is highly sensitive to changes in coordination number and geometry of the Mo active site [25,26]. We note that mutation of the W90 residue in BSO resulted in changes in the *g*-values of the X-band EPR spectrum compared to native enzyme and a shift from a rhombic signal-giving species to a near-axial species [22]. Although the nature of these changes is difficult to interpret it will be interesting to investigate the EPR spectroscopic properties of the W116F mutant of DMSO reductase and compare it to the known signal-giving species associated with native enzyme [24,27].

The variation in pH dependence of the $Mo^{V/IV}$ couple going from native to the W116F mutant enzyme again implicates W116 in H-bonding with the terminal oxygen ligand. As mentioned above, the H-bonding interaction presumed to remain in the native enzyme upon reduction to Mo^{IV} is absent in the W116F mutant. Therefore, the hydroxo-Mo^{V} moiety is able to accept a second proton upon reduction to the tetravalent state. The apparent break in the W116F mutant $Mo^{V/IV}$ pH profile allows an estimate of the putative hydroxo/aqua-Mo^{IV} protonation constant ($pK_a \approx 9$), which is evidently much lower ($pK_a < 5$) in the native enzyme due to the competing influence of the W116 H-bond donor.

In conclusion, our data indicate that W116 plays a pivotal role in maintaining the structure of the Mo active site in DMSO reductase. We are currently pursuing a crystal structure of the W116 mutant enzyme and, together with further spectroscopic studies (e.g. Raman spectroscopy, EPR spectroscopy), this structural information will aid interpretation of our electrochemical and kinetic data.

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