Optical Transitions of Molybdenum(V) in Glycerol-Inhibited DMSO Reductase from *Rhodobacter sphaeroides*

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The DMSO reductases from *Rhodobacter sphaeroides*[1] and *Rhodobacter capsulatus*[2] are unique among molybdenum oxotransferases in that they contain the molybdenum cofactor as their sole prosthetic group. This has facilitated observation of the optical bands associated with the diamagnetic Mo(VI) and Mo(IV) forms of the molybdenum center in situ.[1] Although the intermediate Mo(V) redox state has been extensively characterized by EPR spectroscopy,[3] the absorption characteristics of Mo(V) forms are more difficult to assess since the Mo(V) state is present only in substoichiometric amount. For example, in *R. sphaeroides* DMSO reductase, the Mo(VI)/Mo(V) and Mo(V)/Mo(IV) redox couples are separated by <20 mV at room temperature, which results in no more than 25% of the Mo(V) form during EPR-monitored redox titrations.[1] Here we report detection and preliminary assignment of the optical transitions of the Mo(V) center in glycerol-inhibited *R. sphaeroides* DMSO reductase using the combination of absorption, CD, and variable-temperature magnetic CD (VTMCD) spectroscopies. This inhibited form permits spectroscopic investigations of an oxygen-insensitive form of the enzyme that is almost completely in the Mo(V) redox state. Using a simple theoretical model, the optical transitions revealed by VTMCD studies in the UV/visible region are assigned to π-dithiolene → Mo(V) charge-transfer transitions.

The purity, activity, metal content, and absorption characteristics of the DMSO reductase from *R. sphaeroides* used in this work were identical to those previously described.[1] The glycerol-inhibited Mo(V) form of the enzyme was prepared by anaerobic reduction with a stoichiometric amount of reduced benzyl viologen followed by addition of 50% (v/v) glycerol. The resulting sample was inactive and exhibited an almost axial Mo(V) EPR signal, $g = 1.99$, 1.98, and 1.96 (inset, Figure 1), that accounted for 0.8 spins/Mo. While the g-values are very similar to those observed for the transient Mo(V) form produced in the absence of glycerol,[1] the almost isotropic proton hyperfine coupling attributed to a Mo–OH group is not observed. This inhibited form of the enzyme is stable in air for at least 24 h at 4 °C and is unaffected by repeated freezing and thawing cycles or by dialysis to remove excess glycerol. The properties of this Mo(V) form of DMSO reductase, particularly the inertness toward oxidation and reduction and the EPR parameters, are very similar to those of glycol- or glycerol-inhibited desulfo xanthine oxidase,[4] where they have been attributed to replacement of bound hydroxide and a weakly coordinated anion or buffer ion by bidentate $-OCHRCH_2O-$ (R = H, CH_2OH).[3] Hence, by analogy, the molybdenum coordination in glycerol-inhibited DMSO reductase most likely involves two Mo–S bonds from the dithiolene side chain of the pterin cofactor,[5,6] one Mo=O bond, and two Mo–O bonds from coordinated glycerol, with the possibility of a sixth O/N/S ligand from a protein side chain.

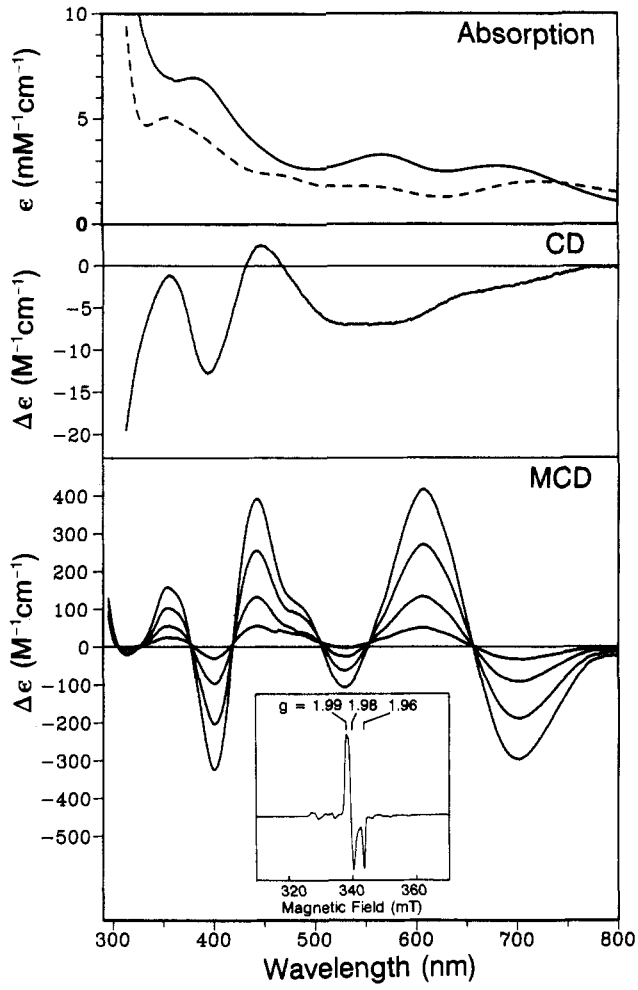

Figure 1. UV–visible absorption, CD, VTMCD, and EPR spectra of the glycerol-inhibited Mo(V) form of DMSO reductase from *R. sphaeroides*. Upper panel: Room-temperature absorption spectra of the native Mo(VI) form (dashed line), 750 μM in 50 mM tricine buffer, pH 7.5, and the glycerol-inhibited Mo(V) form (solid line) prepared by reduction with a stoichiometric amount of reduced benzyl viologen followed by addition of 50% (v/v) glycerol to give a final concentration of 260 μM. Middle panel: Room CD spectrum of the glycerol-inhibited Mo(V) form used for absorption studies. Lower panel: VTMCD spectra of the glycerol-inhibited Mo(V) form used for absorption and CD studies. Spectra were recorded with a magnetic field of 4.5 T at temperatures of 1.61, 4.22, 9.6, and 27.2 K. All bands increase in intensity with decreasing temperature. The MCD $\Delta\epsilon$ values are based on the Mo(V) concentration, 210 μM, as assessed by quantitation of the EPR signal under nonsaturating conditions. The inset shows the X-band EPR spectrum of the MCD sample recorded at 150 K with 1.0-mW microwave power and 0.63-mT modulation amplitude.

Formation of the glycerol-inhibited Mo(V) form of DMSO reductase is accompanied by marked changes in the UV/visible absorption spectrum compared to that of the native Mo(VI) form, as shown in Figure 1. The room-temperature absorption spectrum consists of broad bands centered at 681, 567, and 379 nm, and the molar extinction coefficients, 2700, 3300, and 7000 M^{-1} cm^{-1},

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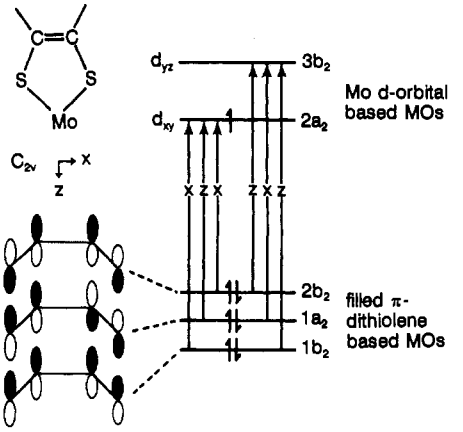

Figure 2. Partial qualitative MO diagram and predicted polarizations for π-dithiolene → Mo(V) charge-transfer transitions within a Mo–dithiolene fragment under effective C_{2v} symmetry. The transition polarizations relate to the axis system shown.

respectively, indicate that these bands arise from ligand → Mo(V) charge-transfer transitions. VTMCD studies afford a more detailed analysis of the optical transitions since only the paramagnetic Mo(V) form will give rise to temperature-dependent bands (*C*-terms), and these can be positive or negative in sign.[7] The MCD spectrum comprises six temperature dependent bands, three positive *C*-terms at 606, 442, and 358 nm and three negative *C*-terms at 701, 530, and 400 nm (Figure 1), and MCD magnetization studies[8] confirm that all six transitions arise from an $S = {}^1/_2$ ground state with $g_{av} = 1.98$ (data not shown).

Recent VTMCD studies of *R. capsulatus* DMSO reductase revealed the optical transitions associated with the proton-split Mo(V) EPR signal (0.06 spins/Mo) of the uninhibited enzyme.[9] The spectra are weaker but similar in form to those shown in Figure 1, except that the corresponding bands are shifted to higher energy by approximately 900 cm^{-1} and the middle negative band is less pronounced. The two lowest energy, oppositely-signed MCD bands were assigned to perpendicularly-polarized π-dithiolene → Mo(V) charge-transfer transitions of the Mo–dithiolene fragment under the effective C_{2v} symmetry.[9] While definitive assignments await VTMCD studies of appropriate Mo(V) model complexes and resonance Raman studies of Mo(V) forms of DMSO reductase, the following discussion demonstrates that all six bands in the MCD spectrum of glycerol-inhibited DMSO reductase can be rationally assigned to π-dithiolene → Mo(V) charge-transfer transitions.

The predicted order of energy and the symmetry labels for the filled dithiolene π-molecular orbitals of the Mo–dithiolene fragment under effective C_{2v} symmetry are shown in Figure 2. Although we have no *a priori* knowledge of the relative energies of the Mo d-orbitals, the alternating signs of the temperature-dependent MCD bands greatly limit the possible assignments, since only perpendicularly polarized transitions will give rise to oppositely signed *C*-terms. Only transitions from each of the filled dithiolene π-orbitals to the Mo d_{xy} and d_{yz} orbitals can give rise to the observed positive–negative–positive and negative–positive–negative patterns of MCD *C*-terms; see Table I and Figure 2. Two additional pieces of evidence support the assignments given in Table I. First, the room-temperature CD spectrum, see Figure 1, consists of three broad negative bands with maxima at 700 (shoulder), 540, and 395 nm, corresponding to each of the positive VTMCD bands. This is consistent with the assignments in Table I, since only the *x*-polarized transitions are magnetic dipole allowed and therefore expected to exhibit CD under the parent C_{2v} symmetry. Second, the energy difference between the pair of transitions originating from each π-dithiolene orbital is almost constant, indicating that the $(d_{xy})^2$ and $(d_{xy})^1(d_{yz})^1$ configurations are separated in energy by approximately 8500 cm^{-1}. Since interelectron repulsion is expected to be greater for the $(d_{xy})^2$ excited-state configuration, the splitting between d_{xy} and d_{yz} must be >8500 cm^{-1}. A weak negative MCD band is observed at ~11 000 cm^{-1} (data not shown), and this is tentatively attributed to the lowest energy, $(d_{xy})^1 \rightarrow (d_{yz})^1$, Mo(V) d–d band.

Table I. Assignment of the MCD Spectrum of Glycerol-Inhibited DMSO Reductase

transition			
one-electron	energy states	polarization	energy, cm^{-1} (nm)
$2b_2(\pi) \rightarrow 2a_2(d_{xy})$	$^2A_2 \rightarrow {}^2B_2$	*x*	14 260 (701)
$1a_2(\pi) \rightarrow 2a_2(d_{xy})$	$^2A_2 \rightarrow {}^2A_2$	*z*	16 500 (606)
$1b_2(\pi) \rightarrow 2a_2(d_{xy})$	$^2A_2 \rightarrow {}^2B_2$	*x*	18 870 (530)
$2b_2(\pi) \rightarrow 3b_2(d_{yz})$	$^2A_2 \rightarrow {}^2A_2$	*z*	22 620 (442)
$1a_2(\pi) \rightarrow 3b_2(d_{yz})$	$^2A_2 \rightarrow {}^2B_2$	*x*	25 000 (400)
$1b_2(\pi) \rightarrow 3b_2(d_{yz})$	$^2A_2 \rightarrow {}^2A_2$	*z*	27 930 (358)

Although we cannot rule out the possibility of that some or all of the higher energy bands arise from charge-transfer transitions involving the oxo group, the σ-dithiolene orbitals, or a coordinated cysteinyl-S, the internal consistency of the assignments argues in favor of the visible absorption bands of glycerol-inhibited Mo(V) DMSO reductase arising almost exclusively from π-dithiolene → Mo(V) charge-transfer transitions. Such transitions should be common to all molybdopterin and tungstopterin enzymes, and recent MCD studies of a W(V) form of *P. furiosus* aldehyde oxidoreductase confirm this prediction.[10] The MCD spectrum comprises an analogous pattern of temperature-dependent bands with each corresponding transition shifted up in energy by ~4000 cm^{-1}, consistent with the expected higher energy of the W 6d orbitals. The MCD results therefore support coordination of Mo or W in these enzymes by the dithiolene side chain of the pterin cofactor.

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