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Detection of the optical bands of molybdenum(V) in DMSO reductase (*Rhodobacter capsulatus*) by low-temperature MCD spectroscopy

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Dimethylsulphoxide (DMSO) reductase from *R. capsulatus* contains a molybdenum-pterin cofactor at its active site. As prepared the molybdenum is in the 6+ oxidation state, devoid of EPR signals. Stepwise reduction generates an EPR signal characteristic of Mo(V) having hyperfine coupling to a single proton and integrating to less than 25% of the total molybdenum. The low temperature MCD spectrum shows oppositely signed bands between ~550–700 nm. These bands are assigned as dithiolene-to-Mo(V) charge transitions. A simple theoretical model can satisfactorily account for the bands in the MCD spectrum. No evidence is found for cysteine coordination to Mo(V).

Molybdenum; Magnetic circular dichroism; Electron paramagnetic resonance; Dimethylsulphoxide reductase

1. INTRODUCTION

Dimethylsulphoxide (DMSO) reductase has been isolated from a wide variety of bacteria including *Escherichia coli* [1], *Rhodobacter capsulatus* [2] and *Rhodobacter sphaeroides* [3]. In *E. coli* the enzyme is membrane- associated and contains both molybdenum cofactor at the catalytic centre and iron sulphur clusters of the [4Fe–4S] type. By contrast, the DMSO reductase from *R. capsulatus* and *R. sphaeroides* is a soluble periplasmic enzyme ($M_r \approx 82{,}000$) and contains only a molybdenum cofactor. The extractable cofactor, molybdopterin, consists of a pterin ring linked to a dithiolene side-chain which probably chelates the molybdenum ion [4]. The cofactor in *R. sphaeroides* DMSO reductase is also covalently linked through a pyrophosphate bond to guanosine monophosphate [5]. Although no X-ray structure is available of any molybdoenzyme there is considerable spectroscopic data including EPR of the Mo(V) state [3,6] and EXAFS studies [7]. Optical methods have not been widely applied to the study of molybdoenzymes since the presence of additional chromophores such as flavin, iron–sulphur clusters or haem groups obscures the molybdenum transitions. Attempts to overcome this problem have used either preparations of an enzyme from which the interfering domain has been cleaved [8] or low-temperature magnetic circular dichroism (MCD) spectroscopy which detects only the paramagnetic Mo(V) centre [9]. For these reasons DMSO reductases from *R. capsulatus* and *R. sphaeroides* provide an interesting molybdoenzyme for spectroscopic study. We report the low-temperature MCD spectra of DMSO reductase (*R. capsulatus*) which identify optical transitions of the Mo(V) state that can be assigned to dithiolene-to-Mo(V) charge-transfer transitions.

Abbreviations: EPR, electron paramagnetic resonance; MCD, magnetic circular dichroism; EXAFS, extended X-ray absorption fine structure; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; EDTA, ethylene diaminetetraacetic acid; Tris, Tris(hydroxymethyl)aminomethane; Bicine, *N,N*-bis(2-hydroxyethyl)-glycine.

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2. MATERIALS AND METHODS

DMSO reductase was prepared from cells of *R. capsulatus* essentially as described previously [2]. Two enzyme preparations were used for this work with specific DMSO reductase activities of 30.0 units · mg^{-1} and 46.3 units · mg^{-1} [2]. Both preparations were judged pure after analysis by SDS-PAGE. Protein determination was by the method of Bradford [10].

Room temperature absorption spectra were recorded on a SLM Aminco DW-2000 spectrophotometer, in the split-beam mode, interfaced to a Viglen II PC for data collection and manipulation. EPR spectra were recorded at X-band frequency with a Bruker ER-200D spectrometer equipped with an ESR-9 flow cryostat (Oxford Instruments PLC, Osney Mead, Oxford). EPR spin concentration was determined by the method of Aasa and Vanngård [11], using 1 mM Cu-EDTA as a standard. MCD spectra were measured using a JASCO J-500D spectropolarimeter interfaced to an IBM PC-AT for data collection and manipulation. The 5 Tesla magnetic field was generated by a SM4 split coil superconducting magnet (Oxford Instruments plc). $\Delta\varepsilon$ values ($=\varepsilon_L - \varepsilon_R$, the molar extinction coefficients for left and right circularly polarised light) are not normalised for magnetic field and are based on the Mo(V) molarity determined by spin integration of the EPR signal.

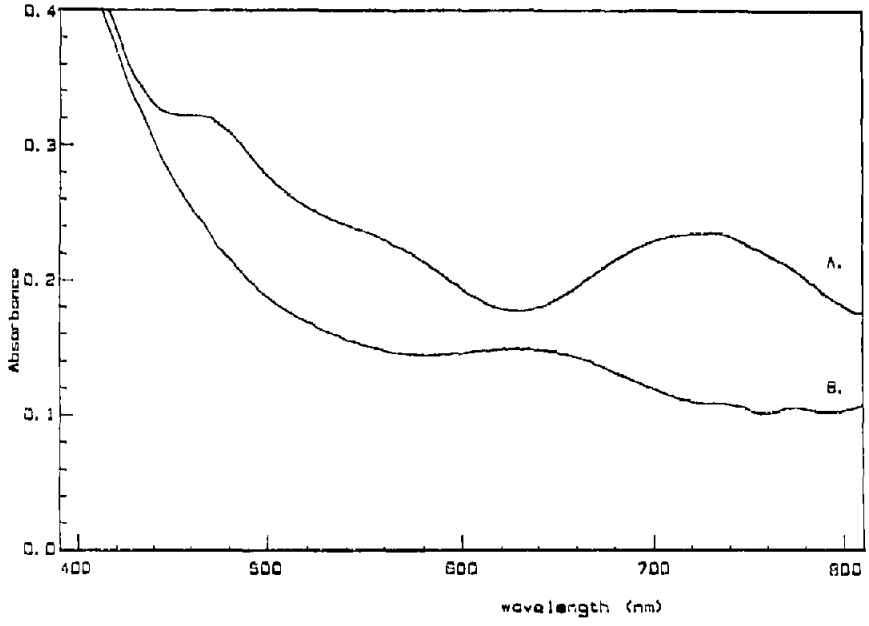

Fig. 1. Room temperature absorption spectra of *R. capsulatus* DMSO reductase, 13.5 mg · ml^{-1} in 50 mM Tris, pH 8.0. (A) Oxidised (native) enzyme. (B) Dithionite reduced enzyme. Pathlength is 1 cm.

3. RESULTS AND DISCUSSION

The lower activity preparation of the enzyme (30.0 units · mg^{-1}) exhibited a weak EPR signal in the native state due to Mo(V) which when integrated accounted for less than 1% of the total molybdenum ion content of the protein. This EPR signal had g-values of 1.99, 1.98 and 1.96 and was devoid of hyperfine coupling. However, the higher activity enzyme preparation (46.3 units · mg^{-1}) lacked Mo(V) EPR signals in the oxidised state. This enzyme was partially reduced for MCD spectroscopy either by the anaerobic addition of sodium dithionite solution or by the addition of methyl viologen which had been electrochemically reduced and hence was free from dithionite or its oxidation products. Both methods of reduction generated an EPR signal characteristic of Mo(V). Each one of the rhombic trio of g-values is split due to coupling to a single nucleus of spin 1/2 (see inset of Fig. 2). Exchange of the water solvent for D_2O removed the hyperfine splitting showing that the coupling is due to a labile proton (data not shown).

Partial reduction of DMSO reductase generates a mixture of molybdenum oxidation states Mo(VI), Mo(V) and Mo(IV). This is to be expected because in the case of the DMSO reductase *R. sphaeroides* the mid-point potentials are +144 mV for Mo(VI)/Mo(V) and +160 mV for the Mo(V)/Mo(IV) couple [3]. In our experiments integration of the *R. capsulatus* EPR signals showed that no more than 25% of the Mo(V) oxidation state can be generated. As a consequence it is not possible to measure the absorption spectrum of a sample of enzyme containing only that oxidation state. The absorption spectrum of oxidised DMSO reductase (Fig. 1A), containing only Mo(VI) ion, shows a broad peak at 720 nm ($\varepsilon \sim 2000$ M^{-1} · cm^{-1} [3]) followed by a series of peaks of increasing intensity towards shorter wavelengths. This extinction coefficient implies a ligand-to-Mo(VI) charge-transfer (CT) origin for the 720 nm band and those to shorter wavelength. The only donor atoms capable of giving rise to a CT band at such a long wavelength are either the sulphur ligands of the dithiolene side-chain of the cofactor or the thiolate group of cysteine. On complete reduction the absorption spectrum of the Mo(IV) species is obtained (Fig. 1B). The extinction coefficient of the longest wavelength intense peak, 640 nm, $\varepsilon = \sim 1000$ M^{-1} · cm^{-1}, suggests a ligand-to-Mo(IV) CT assignment for this band.

The MCD spectra at 1.6 K and 4.2 K and a magnetic field of 5 Tesla of the enzyme partially reduced by sodium dithionite are shown in Fig. 2. The sample has glycerol added to a level of 50% (v/v) in order to form a glass suitable for optical measurement at low temperature. This solvent caused no changes in the EPR spectrum. MCD magnetisation studies as in [12] at selected wavelengths confirm that the bands observed arise from a paramagnetic species with spin $S = 1/2$ and $g_{\text{average}} \approx 2.0$ (not shown).

The low temperature MCD spectrum is dominated by transitions arising from the Mo(V) oxidation state since this is the only paramagnetic species present. The observed differential extinction coefficients suggest that the transitions observed in the wavelength range 300–700 nm are likely to be ligand-to-Mo(V) CT transitions. The absorption and MCD spectra of a variety of Mo(V)

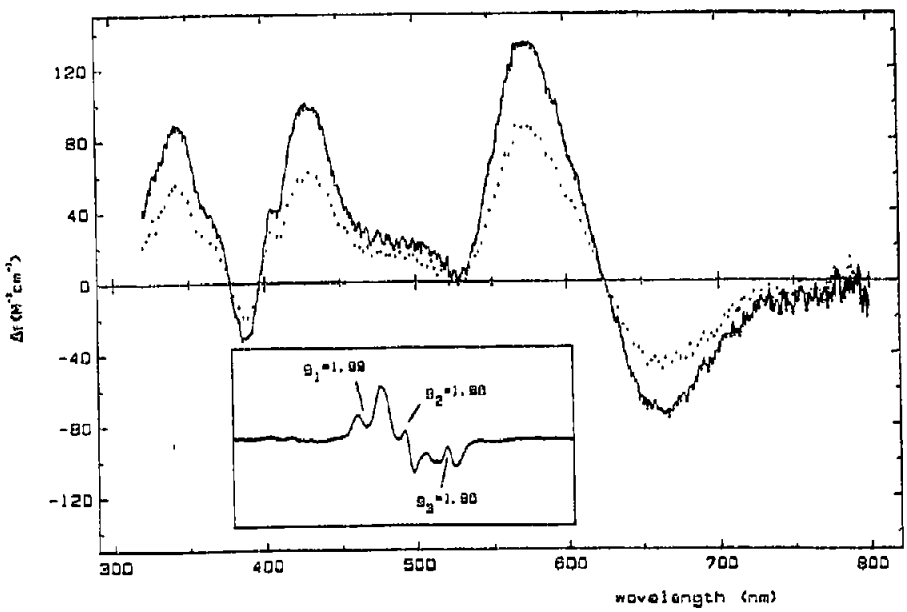

Fig. 2. 1.6 K (———) and 4.2 K (····) 5 Tesla MCD spectra of *R. capsulatus* DMSO reductase, in 150 mM Bicine, pH 7.8, diluted to 50% (v/v) with glycerol, final protein concentration is 145 mg · ml^{-1}. The Mo(V) concentration is 87 μM, i.e. 6% of the total Mo content. Inset shows EPR spectrum of the sample used for MCD, measured at 9.39 GHz, 20 dB power and 150 K.

model compounds in which the metal ion is coordinated by alkyl thiolate ligands show CT bands, thiolate-to-Mo(V), in the wavelength range 500–700 nm with typical $\Delta\varepsilon$ values of 100–400 $M^{-1} \cdot cm^{-1}$ at 1.6 K and 5 Tesla (J.A. Farrar, A. Goodwin, C.D. Garner and A.J. Thomson, unpublished data). Thus the bands between 500 and 700 nm in DMSO reductase undoubtedly arise from the presence of sulphur ligation. The oppositely signed bands, the positive peak at 560 nm and the negative trough at 650 nm, must arise from optical transitions which are polarised perpendicular to one another. It is not known whether the Mo(V) site of DMSO reduc-

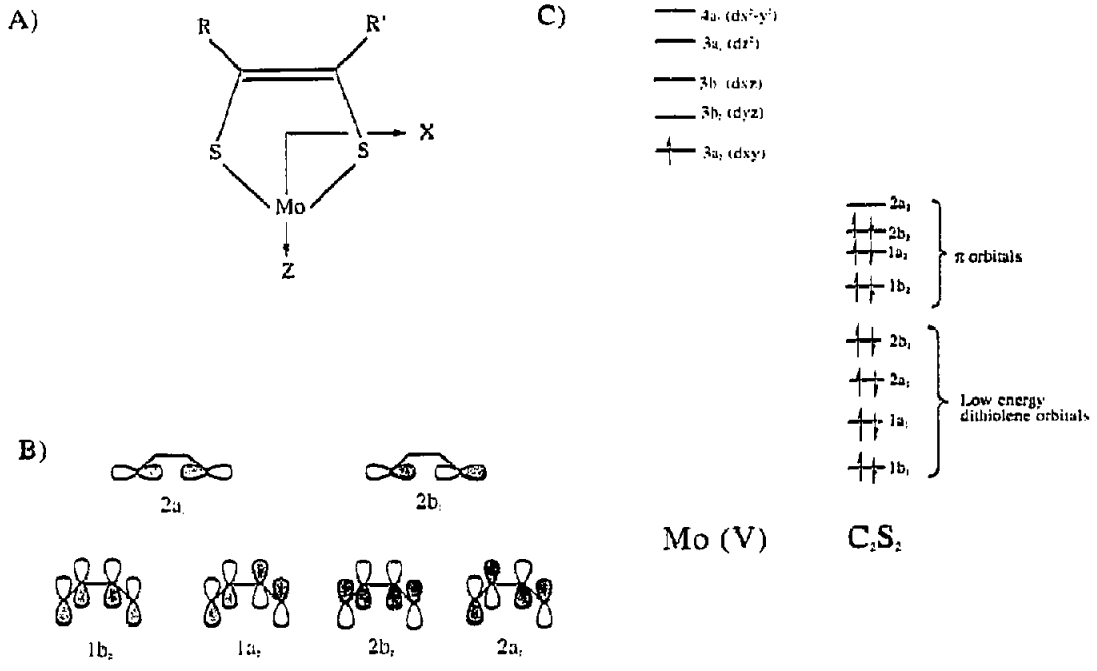

Fig. 3. (A) Molybdenum-dithiolene fragment of molybdopterin showing C_2v symmetry. (B) Linear combinations of p-orbitals in dithiolene that form π molecular orbitals. Shading indicates opposite phase of the orbital to the clear region. (C) Energy level diagram for proposed model of MCD transitions.

Table I

Dithiolene-to-molybdenum(V) CT transitions

Ground state $^2A_2$		
One electron transition	Excited state	Polarisation
$2b_2(\pi) \rightarrow 3a_2$	$^2B_2$	x
$1a_2(\pi) \rightarrow 3a_2$	$^2A_2$	z
$1b_2(\pi) \rightarrow 3a_2$	$^2B_2$	x
$2a_1(\sigma) \rightarrow 3a_2$	$^2A_1$	Forbidden
$2b_1(\sigma) \rightarrow 3a_2$	$^2B_1$	y

tase contains ligation by cysteine thiolate. However, resonance Raman spectra of the Mo(VI) oxidation state identify vibrations due to the C=C stretch of the dithiolene ligand and the Mo–S stretches [13]. This gives clear evidence of dithiolene–molybdenum coordination.

A number of low energy CT transitions from the π orbitals of the unsaturated dithiolene group are expected. We therefore examine a theoretical model of a Mo(V)–dithiolene fragment in order to assign the observed transitions. Fig. 3A shows such a fragment of C_{2v} symmetry and defines a coordinate system. The sulphur $3p_y$ and carbon $2p_y$ orbitals are conjugated in a π-system extending over four atoms of the dithiolene ligand. The resulting π-orbitals are either symmetric (b_2) or antisymmetric (a_2) with respect to reflection in the vertical mirror plane, yz. The sulphur p_x (and p_z) orbitals are not conjugated to the dithiolene π-system but do form a symmetric (a_1) and antisymmetric (b_1) pair of orbitals (Fig. 3B). The highest energy orbitals are likely to be those of π character.

The selection rules for CT transitions from these orbitals into the lowest energy d-orbital of Mo(V) depend upon the orientation of the latter orbital with respect to the dithiolene molecular framework. This is not known. However the orbital of interest contains the unpaired d-electron giving rise to the EPR signal which shows that it is non-degenerate. Under the point group C_{2v} this orbital can have one of the following symmetry labels, a_1 (d_{z^2}, $d_{x^2-y^2}$), a_2 (d_{xy}), b_1 (d_{xz}) or b_2 (d_{yz}). Inspection of the selection rules for CT transitions from the three highest filled π-orbitals $b_2(\pi)$, $a_2(\pi)$, and, $b_2(\pi)$, to each of these metal d-orbitals shows that only transitions to $d_{xy}(a_2)$, or $d_{yz}(b_2)$ orbitals give rise to oppositely polarised optical transitions as required by the MCD spectrum. We cannot choose between these two assignments for the ground state orbital. However, the conclusions are illustrated in Fig. 3 and Table I for the case of the unpaired electron in the d_{xy} orbital. The two oppositely signed transitions are then assigned as x- and z-polarised CT transitions, $2b_2(\pi) \rightarrow 3a_2(d_{xy})$ and $1a_2(\pi) \rightarrow 3a_2(d_{xy})$, respectively. The third transition expected, namely $1b_2(\pi) \rightarrow 3a_2(d_{xy})$, will generate an MCD band of the same sign as the x-polarised transition. This may be the positive peak at 420 nm in the MCD spectrum. At higher energy, CT transitions are expected from the dithiolene sulphur-orbitals, which form σ-bonds to Mo(V). However, at higher energy transitions from other molybdenum ligands such as an oxo group or histidine ligands may contribute to the complexity of the spectrum.

We cannot categorically rule out the presence of thiolate ligation to molybdenum(V) from cysteine residues. However, given the evidence for dithiolene coordination and the satisfactory assignment of the MCD spectrum based upon dithiolene-to-Mo(V) transitions alone it seems unlikely that cysteine ligands are present. We would expect thiolate-to-Mo(V) CT transitions to lie in the region 500–700 nm and to complicate the MCD spectrum considerably. EXAFS measurements should enable this conclusion to be tested.

Comparison of the MCD spectrum of the Mo(V) centre in partially reduced DMSO reductase (*R. capsulatus*) with that of the desulpho inhibited form of xanthine oxidase [9] shows some similarities between the two over the wavelength range 300–500 nm. The latter has positive peaks at 330 nm, 440 nm and 510 nm. It was not possible to obtain a spectrum of xanthine oxidase at wavelengths longer than ~550 nm, the region in which the dithiolene(π)-to-Mo(V) CT bands have been identified in DMSO reductase. It is important to collect the MCD spectra of Mo(V) oxidation states from a wider range of molybdenum centres in enzymes in order to build upon this partial model of the centre in DMSO reductase.

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