Detection by low-temperature magnetic circular-dichroism spectroscopy of optical absorption bands due to molybdenum(V) in the form of xanthine oxidase giving the Desulpho Inhibited e.p.r. signal

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The magnetic circular-dichroism (m.c.d.) spectra in the temperature range 1.5–100 K and the electronic absorption spectra at 4.2 and 295 K were measured for a number of desulpho xanthine oxidase derivatives. There were no significant differences between the absorption spectra that could be attributed to molybdenum. However, the visible-region m.c.d. spectrum of the ethanediol-treated metalloprotein (which gives rise to the Desulpho Inhibited e.p.r. signal) contained features assignable to Mo(V) absorption bands. This is the first report of the detection of optical bands of Mo(V) in an enzyme in the presence of other chromophoric centres.

INTRODUCTION

Much structural information on molybdenum centres in enzymes has been deduced from studies by e.p.r. of molybdenum in the quinquevalent state and by extended X-ray-absorption fine structure (Bray, 1980, 1984; Cramer, 1983; Bray & George, 1985). Though for all known molybdoenzymes the catalytic reaction takes place at the molybdenum, other centres, such as iron-sulphur, flavin or haem, are invariably present in the enzyme molecules also. Since these additional centres all have much more intense absorption spectral characteristics in the near-u.v.-visible region than does molybdenum, the contribution of this metal to the spectrum has not been determined in any case. This is unfortunate, since the evaluation of such spectral features would be expected to provide considerable new insight into the structure and bonding of the metal at the active sites.

Low-temperature m.c.d. spectroscopy offers in principle a way of locating the optical absorption bands of molybdenum in paramagnetic oxidation states such as Mo(V). This is possible because the m.c.d. spectra of paramagnetic species are temperature-dependent, increasing in intensity dramatically upon cooling to cryogenic temperatures. Since the m.c.d. bands of diamagnetic chromophores are invariant to temperature, the spectrum at liquid-He temperature is dominated by the contributions from the paramagnetic species present.

Xanthine oxidase is the most extensively studied of the molybdoenzymes. In addition to the molybdenum, [2Fe-2S]-core clusters and FAD centres are also present (Bray, 1975). A peculiarity of this enzyme is that, as normally isolated and studied, it is a mixture of two molybdenum-containing forms, namely the active enzyme and a catalytically inactive degradation product, desulpho xanthine oxidase. The active form can be converted into the desulpho form by treatment with cyanide, which liberates a sulphur atom from the enzyme

and forms thiocyanate (Massey & Edmondson, 1970). After dithionite reduction, addition of ethanediol to the desulpho form leads to a new non-functional form, giving the so-called Desulpho Inhibited Mo(V) e.p.r. signal, having about 50% of the total molybdenum in the quinquevalent state (Lowe *et al.*, 1976; this e.p.r. signal was originally referred to as Resting II). In sharp contrast with other Mo(V) e.p.r. signals, the Desulpho Inhibited signal is resistant to both oxidation and reduction. Consequently, air oxidation of the reduced, ethanediol-treated, desulpho xanthine oxidase results in a form of the enzyme in which Mo(V) is the only paramagnetic species present, the [2Fe-2S]-clusters and the FAD being diamagnetic. Of the many e.p.r. signals observable from different molybdoenzymes, the Desulpho Inhibited signal from xanthine oxidase is the only one that can readily be obtained in stable form with other paramagnetic centres absent, and for this reason it was chosen for initial m.c.d. studies. Henceforth in the present paper we refer to this form of the enzyme as 'desulpho inhibited xanthine oxidase'. This is not to be confused with the 'Desulpho Inhibited signal' (with capitals) of previous communications [e.g. Lowe *et al.* (1976), which refers to the Mo(V) e.p.r. spectrum].

As a first step in the investigation of the optical properties of molybdenum in metalloproteins, we report herein the extraction of the paramagnetic component of the m.c.d. spectrum of desulpho inhibited xanthine oxidase.

MATERIALS AND METHODS

Xanthine oxidase derivatives were prepared from the enzyme (Hart *et al.*, 1970; Bray, 1982) according to previously published methods, namely desulpho xanthine oxidase (Massey & Edmondson, 1970), deflavo desulpho xanthine oxidase (Komai *et al.*, 1969) and desulpho

Abbreviation used: m.c.d., magnetic circular dichroism.

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inhibited xanthine oxidase (Lowe *et al.*, 1976). Samples for low-temperature work were prepared in 50 mM-Bicine buffer, pH 8.2. In order to obtain optically transparent glasses, glycerol was added to 50% (v/v). AnalaR-grade reagents (BDH Chemicals) were used throughout.

Variable-temperature m.c.d. measurements were carried out with a Jasco J-500D spectropolarimeter and split-coil superconducting magnet (SM-4; Oxford Instrument Co.). Spectra are expressed at a given field and temperature in units of $\Delta\epsilon = \epsilon_L - \epsilon_R$, where ϵ_L and ϵ_R are the molar absorption coefficients for the absorption of left and right circularly polarized light respectively. E.p.r. spectra were recorded in an X-band spectrometer (Bruker ER-200D), interfaced with a mini-computer (Aspect 2000) and fitted with a flow-cryostat (ESR-900; Oxford Instruments Co.). Quantification of the Mo(V) e.p.r. signal was performed by double integration, with Cu(II)-EDTA as a spin integration standard. Absorption spectra were measured at 4.2 K in the split-coil magnet on the same samples as were used for m.c.d. studies. The spectrophotometer was a Cary 17D instrument.

RESULTS AND DISCUSSION

The absorption spectra at 4.2 and 295 K of desulpho inhibited xanthine oxidase are shown in Fig. 1 together with the 4.2 K spectrum of deflavo desulpho xanthine oxidase. The absorption spectra of the oxidized active and desulpho forms of xanthine oxidase (not shown) are a good approximation indistinguishable from that of desulpho inhibited xanthine oxidase at the same temperature. Certainly at 4.2 K any differences between the absorption spectra of these forms of the enzyme are within the limits of experimental error on their measurement. It is clear from the data that the spectral features in absorption arise predominantly from two kinds of chromophore. The deflavo enzyme lacks a broad feature between 400 and 500 nm that peaks at about 440

Fig. 1. Absorption spectra of xanthine oxidase derivatives

Spectra: desulpho inhibited form at 295 K (—) and 4.2 K (·····), 370 μ M enzyme concentration, 1 mm pathlength; deflavo desulpho form at 4.2 K (----), 94 μ M enzyme concentration, 1 mm pathlength. Corrections for baseline curvature in the 4.2 K data have not been applied.

Fig. 2. C.d. and m.c.d. spectra of xanthine oxidase derivatives

Spectra at 4.2 K: desulpho inhibited form c.d. (—) and m.c.d. at 5 T (·····), 370 μ M enzyme concentration, 1 mm pathlength; desulpho form m.c.d. spectrum at 5 T (----), 360 μ M enzyme concentration, 1 mm pathlength.

and 470 nm (Fig. 1). This is the absorption due to the flavin moiety. The deflavo enzyme spectrum has maxima or inflexions at approx. 420, 470, 550 and 700 nm, which we assign to the oxidized [2Fe-2S] clusters. The low-temperature absorption spectrum of the oxidized [2Fe-2S] ferredoxin from *Spirulina maxima* is similar, having maxima at approx. 420 and 470 nm (Thomson *et al.*, 1977).

Fig. 2 shows the 4.2 K c.d. and m.c.d. spectra of desulpho inhibited xanthine oxidase (continuous line and dotted line respectively), together with the m.c.d. spectrum of the desulpho form. The natural c.d. of oxidized active, desulpho and deflavo forms of the enzyme at low temperature were found to be very similar to that of desulpho inhibited xanthine oxidase. Comparison with the c.d. spectra of oxidized [2Fe-2S]-containing ferredoxins of the plant type (Thomson *et al.*, 1977; Stephens *et al.*, 1978) reveals that the major contribution is from centres of this kind. The same is true of the m.c.d. spectra of Fig. 2, which are dominated by features assignable to oxidized [2Fe-2S] clusters. However, there is clearly a small but significant difference between the m.c.d. spectrum of oxidized desulpho xanthine oxidase and that of the desulpho inhibited derivative.

Desulpho xanthine oxidase in the oxidized state does not give rise to any e.p.r. signals at either 10 or 77 K. This absence of paramagnetic species was confirmed by the m.c.d. spectrum, which was very near temperature-independent between 1.5 and 80 K. On the other hand, the e.p.r. spectrum of desulpho inhibited xanthine oxidase contains signals that are attributable to Mo(V) only (Lowe *et al.*, 1976). Consequently, the differences between the m.c.d. spectra of Fig. 2 may reflect the presence of Mo(V) in the desulpho inhibited form. Given that this is the case, then it should be possible to obtain the required Mo(V) spectrum by subtraction of the m.c.d. spectrum of desulpho xanthine oxidase from that of desulpho inhibited xanthine oxidase at the same temperature and magnetic field strength. However, the component in the m.c.d. spectrum of interest constitutes only a fraction of the total signal detected, and in practice this means that reliable data are difficult to obtain by this method, because differences between samples due to effects such as light-scattering and/or depolarization lead to variation in spectra of magnitude comparable with the differences exhibited in Fig. 2. Nevertheless it should be noted that this method does yield a Mo(V) spectrum (not

Fig. 3. M.c.d. spectra of desulpho inhibited xanthine oxidase

Spectra at 5 T, 370 μM enzyme concentration, 1 mm pathlength: (a) 1.5 K (----), 4.2 K (.....), 30 K (-.-.-) and 80 K (—) (N.B. the underlying natural c.d. has been subtracted from the data); (b) temperature-difference spectrum, 1.5 K data minus 80 K data.

shown) that is qualitatively similar to that obtained by the temperature-difference procedure described below.

The temperature-dependence of the m.c.d. spectrum of desulpho inhibited xanthine oxidase between 1.5 K and 80 K is shown in Fig. 3(a) (where the underlying natural c.d. has been subtracted out). Above 30 K the spectrum is virtually temperature-independent and closely resembles that of the desulpho derivative. At liquid-He temperatures the spectrum becomes strongly temperature-dependent. This behaviour can be attributed to the paramagnetic centre present, i.e. Mo(V). By plotting the difference m.c.d. spectrum between the 1.5 K and 80 K plots, the form of the m.c.d. spectrum of the Mo(V) signal is determined (Fig. 3b), where the intensity scale is expressed in terms of Mo(V) concentration determined by integration of the e.p.r. signal).

The Desulpho Inhibited e.p.r. signal has g -values 1.967, 1.973, 1.980 (Lowe *et al.*, 1976; Fig. 4a). By using the average g -value of 1.97 (and hence treating the system as isotropic) the m.c.d. magnetization curve expected for a paramagnet with a spin $S = \frac{1}{2}$ ground state has been simulated, following Thomson & Johnson (1980), and is given as the continuous line of Fig. 4(b). On this have been superimposed the m.c.d. magnetization properties of the features at 330, 440 and 510 nm, which have been constructed as follows. Temperature-difference spectra of the form of Fig. 3(b) were constructed at several different magnetic field strengths between 0 and 5 T. The intensity of the signals were then plotted against $\beta B/2kT$, where β is the Bohr magneton and k the Boltzmann constant, after Thomson & Johnson (1980). In order that comparisons between the three wavelengths can be made, all data have been normalized to 100% intensity at 5.0 T.

A reasonable fit is seen (Fig. 4b), given that the intensity of the paramagnetic component of the m.c.d. signal is rather less than that of the c.d. signal. The fact that the temperature-dependent components of Fig. 3(b) magnetize in this way does not exclude contributions from either a reduced [2Fe-2S] centre or from reduced flavin, since both have g -values close to 2.0. However, we

can eliminate this possibility, because the e.p.r. spectra down to 10 K (not shown) are devoid of any signals due to either of these species.

Therefore we conclude that the Mo(V) centre in desulpho inhibited xanthine oxidase has optical absorption bands at 330, 440 and 510 nm. The relative intensities of the m.c.d. bands do not imply that the absorption bands necessarily have the same intensity ratios. There appears to be no other useful determination of the position of the optical absorption bands of Mo(V) in any molybdenum enzyme. The absorption spectra of the apparently haem-free molybdenum domain of sulphite oxidase has been recorded (Johnson & Rajagopalan, 1977). However, in the sulphite-reduced oxidation level, which contains Mo(V), the small degree of haem contamination still partially obscured the spectra and makes the unambiguous observation of Mo(V) impossible.

We next turn to consider the question of the possible structural significance of the observation of the optical bands of Mo(V). Here we are limited by the paucity of good spectra data, especially m.c.d., on structurally well-defined monomeric Mo(V) compounds with a wide variety of ligand types (nitrogen, oxygen or sulphur) and geometry (Stiefel, 1977).

Mo(V) contains one unpaired d -electron. The electronic spectrum consists of some relatively weak bands due to the $d-d$ transitions plus more intense transitions which arise from transitions of electrons from orbitals based primarily on the ligands to the empty d -orbitals of the molybdenum, termed charge-transfer bands. For complexes of a given geometry, the charge-transfer bands have an energy dependent on the nature of the ligand from which the electron arises. The more reducing the ligand the lower is the energy of the charge-transfer band. Thus charge-transfer bands from sulphur ligands will be at lower energy than those from oxygen or nitrogen ligands. However, the energy of the charge-transfer band also depends on the energy of the accepting d -orbital relative to that of the donating ligand orbital. Therefore the precise energy of the band will depend, for example,

Fig. 4. (a) E.p.r. spectrum of desulpho inhibited xanthine oxidase and (b) m.c.d. magnetization of the temperature-dependent bands of desulpho inhibited xanthine oxidase

(a) E.p.r. spectrum of desulpho inhibited xanthine oxidase at 125 K, 9.42 GHz, 20 mW power, 0.1 mT modulation amplitude, 148 μM Mo(V) (370 μM enzyme) concentration. (b) M.c.d. magnetization (see the text for explanation) of the temperature-dependent bands of desulpho inhibited xanthine oxidase at 330 nm ($\circ$), 440 nm ($\bullet$) and 510 nm ($\bullet$), 148 μM Mo(V) (370 μM enzyme) concentration, 1 mm pathlength.

on whether there are a number of oxo groups co-ordinated to Mo(V) and, if so, how many.

Inspection of the available data (Stiefel, 1977; Spence, 1983) leads to the conclusion that metal-to-ligand charge-transfer bands in complexes of Mo(V) containing one oxo group lie at energies below 30000 cm^{-1} ($= 333\text{ nm}$) only if a sulphur ligand (or a π -unsaturated ligand such as 8-hydroxyquinoline) is present in addition. The model compound $[\text{MoO}(\text{SPR})_4]^-$ of Hanson *et al.* (1981) has a single broad peak at 600 nm and intense unresolved absorption at wavelengths shorter than 400 nm. The former has been assigned as the charge-transfer transition from a ligand orbital to an orbital involving the Mo=O species. In a complex of lower symmetry or with a smaller number of sulphur ligands these bands will be shifted in energy. For example, in the binuclear analogues $[\text{Mo}_2\text{O}_2(\text{XR})_6(\text{OMe})]^-$ these absorption bands arise at approx. 500 nm (Hanson *et al.*, 1981).

The distinction between charge-transfer and $d-d$ bands

is usually made on the basis of the molar absorption coefficients, ϵ , values of ϵ in the range $1\text{--}100\text{ M}^{-1}\text{ cm}^{-1}$ being assigned to $d-d$ transitions and values of ϵ greater than $10^3\text{ M}^{-1}\text{ cm}^{-1}$ to charge transfer. The m.c.d. spectrum is a measurement of absorption coefficients with left- minus right-circularly polarized light, $\Delta\epsilon$. $\Delta\epsilon$ values cannot be related unambiguously to ϵ values. Although in principle this should be possible, particularly if ground-state g -values of the species under observation are known, in practice the overlap of oppositely signed peaks from unresolved transitions in the m.c.d. spectrum will lead to cancellation of intensity and to a significant lowering of $\Delta\epsilon$ values below that expected. We suspect that such a cancellation of intensity may be occurring in the m.c.d. spectrum of Mo(V) reported in the present paper. Therefore we have no reliable guide to band assignment from the intensities of the $\Delta\epsilon$ values. M.c.d. data are required on monomeric Mo(V) model compounds of well-defined structure. Recent synthetic approaches to modelling mononuclear active sites of molybdoenzymes promise to provide useful spectral (Berg & Holm, 1984). The ability to compare the optical properties of Mo(V) in enzymes with those of structurally defined models should be of great utility.

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