Volume 307, number 2, 233–236 FEBS 11337 July 1992

Spectroscopic identification of the haem ligands of cellobiose oxidase

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Received 6 April 1992; revised version received 12 June 1992

A spectroscopic study of the flavocytochrome *b* enzyme, cellobiose oxidase, employing optical, NMR, EPR and near infra-red MCD techniques, has identified the axial ligands of the b-type haem. These are a histidine and a methionine, and this ligation set is discussed in relation to the functional role of the haem group.

Cellobiose oxidase; Flavocytochrome *b*; Electron paramagnetic resonance; Nuclear magnetic resonance; Magnetic circular dichroism

1. INTRODUCTION

Cellobiose oxidase is one of a group of enzymes secreted by the white-rot fungus *Phanerochaete chrysosporium*. Although the specific role(s) of this enzyme is not well understood it is widely considered to play a part in this microorganism's lignocellulose degrading system [1,2]. The reaction catalyzed by the enzyme is one in which cellobiose is oxidized to cellobionolactone. The enzyme can rapidly donate electrons to quinones but only slowly to oxygen [3].

Cellobiose oxidase is a monomeric glycoprotein, the molecular weight of which has variously been reported to be between 74,400 and 93,000 Da. Recent analysis using laser induced desorption (LID) mass spectrometry has yielded a value of 89,170 Da [4]. The enzyme contains two prosthetic groups, a b-type haem and a flavin (FAD), both of which may be reduced by cellobiose but with very different kinetics [3,5–6]. These groups are located within protein domains which may be separated by papain cleavage of the linking peptide (cf. flavocytochrome b_2 [7]) [8,9]. The flavin domain retains its ability to react with cellobiose and is antigenically very similar to cellobiose quinone oxidoreductase, which can also be isolated from the growth medium of the fungus, suggesting that the latter is in fact a haem-free breakdown product of cellobiose oxidase [10]. The flavin domain also possesses a cellulose binding site and the enzyme retains activity when bound to this polymer [9]. In recent years, increasing information about the relevance of the biological role of cellobiose oxidase has become available [11], and the finding that the enzyme may reduce chelated iron and, hence, possibly initiate Fenton chemistry, has suggested that it may participate in radical reactions leading to cellulose breakdown [12].

Here we report a spectroscopic investigation of cellobiose oxidase in its various redox states, in an attempt to identify the axial ligands of the cellobiose oxidase *b*-type haem. The identity of the haem axial ligands can yield important functional information as the nature of the ligand set may indicate whether the haem has a ligand binding role or a simple electron transfer function, i.e. whether the haem interacts directly with external oxidants such as O_2, or substrates such as cellobiose, or whether it acts as a single electron reductant for some, as yet unknown, substrate (see [13] and references therein). The established procedure for the spectroscopic identification of haem axial ligands involves the combined use of optical absorption, NMR, EPR and near infra-red MCD spectroscopies [13,14]. The characterization of the ligands of *Escherichia coli* cytochrome b_{562} [15] and plant cytochrome *f* [16] provide good examples. Our studies unambiguously reveal that the b-type haem ligation of cellobiose oxidase are histidine and methionine, and this strongly suggests that the haem has an electron transfer function and, like cytochrome *c* which has the same ligand set, acts as a single electron donor. Our results are consistent with the suggestion by Wood and Wood [10] and Kremer and Wood [12] that the b-type haem may have a role in initiating Fenton chemistry.

Abbreviations: D_2O/HOD, deuterium oxide/deuterium hydroxide; ε, extinction coefficient; EPR, electron paramagnetic resonance; FAD, flavin adenine dinucleotide; His, histidine; MCD, magnetic circular dichroism; Met, methionine; NMR, nuclear magnetic resonance.

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2. MATERIALS AND METHODS

Cellobiose oxidase was prepared from *Phanerochaete chrysosporium* by the method described by Jones and Wilson [5]. Enzyme concentrations were determined using $E_{421} = 65{,}200\ M^{-1}\ cm^{-1}$. Flavocytochrome b_2 from *Saccharomyces cerevisiae* was a generous gift from Dr. S.K. Chapman (University of Edinburgh, Scotland) [7]. Cytochrome *c* (equine heart type VI) was purchased from Sigma.

Electronic spectra were recorded on a Hitachi U-2000 spectrophotometer. MCD spectra were recorded on a Jasco J-500 D spectrophotometer, for the wavelength range 300–1,100 nm, and on a home-built circular dichrograph in the range 800–3,000 nm. The magnetic field was provided by a superconducting solenoid (Oxford Instruments) capable of generating a maximum magnetic field of 6 T with a room temperature bore. The sample was held at room temperature. EPR spectra were recorded on an X-band spectrometer (Brukker ER200) with an ESP1600 computer system fitted with a liquid helium flow cryostat (Oxford Instruments; ESR9). NMR spectra were recorded on a Jeol GX-400 400 MHz spectrophotometer. 16K data points were used to acquire spectra over a frequency range of 6,000 Hz, with a 75° pulse width and a pulse delay of 1 s. Gated irradiation was used to reduce the intensity of the HOD peak. To reduce the intensity of the HOD peak, and to allow field-frequency locking, the sample was exchanged into D_2O prior to NMR spectra and was obtained by 3 cycles of concentration and dilution in Amicon concentration devices. Quoted pH values were direct meter readings uncorrected for any isotope effects. External dioxan was used as a chemical shift standard but chemical shifts are reported in parts per million (ppm) downfield from the methyl resonance of 2,2-dimethyl-2-silapentane-5-sulphonate.

3. RESULTS AND DISCUSSION

3.1. *Optical absorption spectra*

The optical absorption spectra of oxidized and reduced cellobiose oxidase are shown in Fig. 1. The appearance of the spectra indicate that the haem is low-spin in both oxidation states [13,17], the Soret peaks are relatively sharp at 421 nm (ferri) and 429 nm (ferro); the α-β bands of the low-spin ferrohaem are clearly defined at 532 and 562 nm; and there is no band with a wavelength maximum in the region of 640 nm. The low-spin character of the haem iron indicates it has two strong-field axial ligands. Furthermore, the weak band at 729 nm in the oxidized cellobiose oxidase spectrum indicates that the haem has a methionine axial ligand ([13] and the references therein, [18]). This band has been assigned either to a charge-transfer transition from the methionine axial ligand into the Fe(III) d-orbital hole or to a porphyrin to Fe(III) charge transfer (see [13] for review, [19]).

3.2. *Magnetic circular dichroism spectra*

The near-infra-red MCD spectrum of the cellobiose oxidase is shown in Fig. 2. The bands in this region originate from porphyrin → Fe(III) charge-transfer transitions and their wavelength maxima are diagnostic of the axial ligation [14,20]. The maximum at 1,870 nm observed for the haem of cellobiose oxidase indicates clearly that it has histidine and methionine axial ligands.

3.3. *Nuclear magnetic resonance spectrum*

The upfield region of the ^1H NMR spectrum of fully reduced cellobiose oxidase is shown in Fig. 3 together with the corresponding regions of the spectra of core flavocytochrome b_2 and cytochrome *c*. These latter proteins have bis-histidine and histidine–methionine ligation, respectively ([13] and references therein, [21]). The signals between 2 −4 ppm arise from methionine coordinated to the haem iron: the three-proton intensity singlet comes from the methionine CH_3 group and the one-proton intensity peaks come from the β- and α-CH protons.

3.4. *Electron paramagnetic resonance spectroscopy*

The EPR spectrum of the ferrihaem of cellobiose ox-

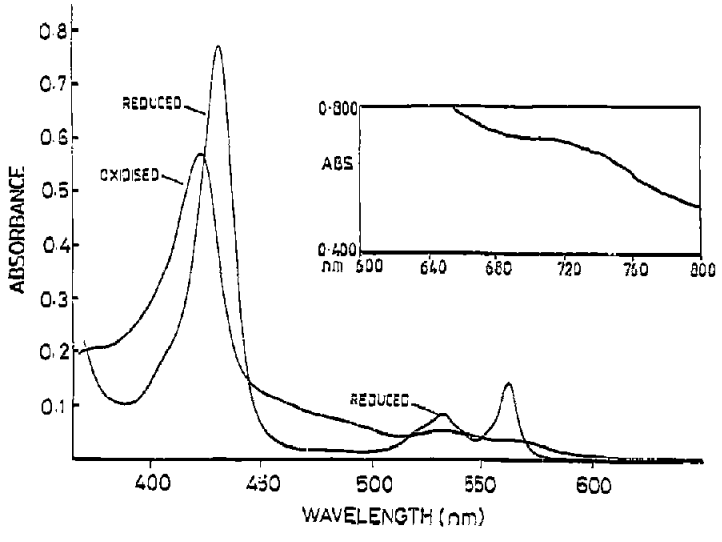

Fig. 1. Optical spectra of oxidized and dithionite reduced cellobiose oxidase; 8.6 μM in protein in 50 mM phosphate buffer, pH 6.25. The insert is a region of the spectrum of 1 mM oxidized cellobiose oxidase in 5 mM phosphate buffer, pH 7.

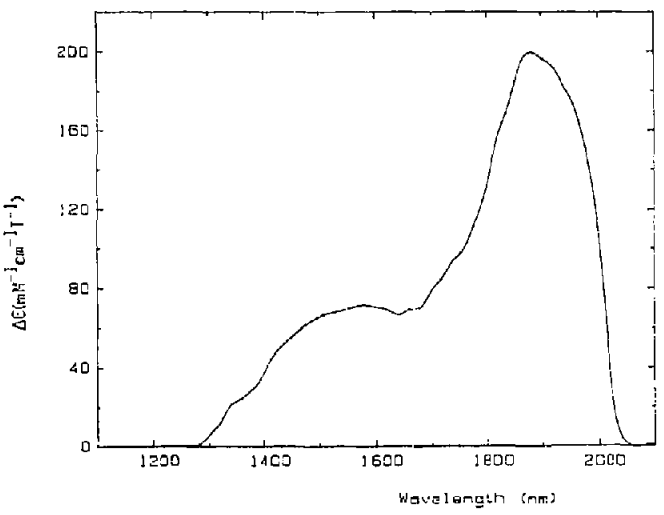

Fig. 2. The near-infra-red MCD spectrum of 1 mM oxidized cellobiose oxidase in 20 mM phosphate buffer, pH 7. The path length was 2 mm. The sample was held at room temperature in a magnetic field of 6 T. The ΔE was expressed per unit magnetic field.

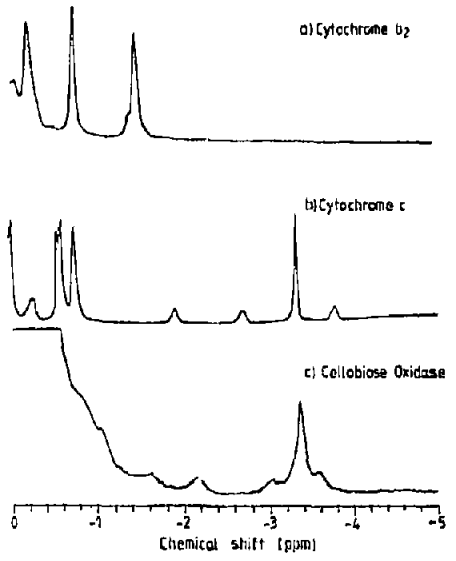

Fig. 3. Regions of the 400 MHz ^{1}H NMR spectra of 1 mM core ferrocytochrome b_2, 5 mM horse ferrocytochrome *c* and 1 mM cellobiose oxidase (dithionite reduced). All samples were in 5 mM phosphate buffer, pH 7.

idase (not shown) is that of a highly axial and non-rhombic system. The distinguishing features of such a system are a ramp-shaped g_z signal with a high value for g_z [22]. In the case of cellobiose oxidase we find $g_z \sim 3.50$, in agreement with the report by Morpeth [2]. This should be compared with the range of g_z values found for most His-Met coordinated haem, of 3.0–3.3 [13]. Gadsby et al. [23] have observed a comparable spectrum to that of cellobiose oxidase for the His-Met coordinated haems of *Azotobacter vinelandii* ferricytochrome c_4, $g_z = 3.64$. They account for this in terms of a model in which the plane of the CH_3-S-CH_2 fragment lies perpendicular to the plane of the trans-His ligand ring. Such an orientation generates a ligand field in which the x and y axes are equivalent as observed. A similar, unusual coordination geometry for cellobiose oxidase, in which the imadazole ring and the methionine side chain of the ligands coordinated to the iron are almost perpendicular, is indicated by our EPR data.

3.5. *General discussion*

The spectroscopic data clearly show that the *b*-type haem of cellobiose oxidase has histidine and methionine ligation in the ferric oxidation state, and that it has two strong-field axial ligands, one of them methionine, in the ferrous state. It is most unlikely that the histidine ligand is replaced during a change in redox state, and therefore we believe that the *b*-type haem has histidine–methionine coordination in both redox states. As far as we are aware, this is the first identification of histidine–methionine ligation in a b-type containing enzyme, and only the second cytochrome *b* to be shown to have such coordination. The other cytochrome *b* with this axial ligation is cytochrome b_{562} from *E. coli* [15].

The findings that the cytochrome *b* of cellobiose oxidase is low-spin in both its oxidized and reduced states strongly suggests an electron-transfer rather than a binding function for this component of the enzyme. This is somewhat at odds with earlier studies of the reaction of reduced cellobiose oxidase with O_2 which suggested that, although the flavin group of the separated domain may be oxidized by oxygen [9], it is the haem group in the intact enzyme which reacts initially with the oxygen [3]. It might have been anticipated that the cytochrome moiety would be high-spin in the ferrous state in order to bind O_2 in the manner, say, of the reversible oxygen carriers, haemoglobin, myoglobin, or the terminal oxidases, cytochrome oxidase or cytochrome *bo*. Oxidation of b-type haem by oxygen is, however, very slow ($k = 0.5\ s^{-1}$) possibly reflecting the dynamics of ligand exchange (O_2 for methionine). Kinetic data, consistent with the above EPR data, shows that O_2 is unlikely to be the oxidant of cellobiose oxidase in vivo [3]. This supports the view of Kremer and Wood [12] who suggest that cellobiose oxidase is a source of Fenton's reagent, reducing Fe(III) to Fe(II) through a single electron transfer, possibly from the haem. The occurrence of flavin radicals which should be generated by the reduction of the b-type haem by flavin have not yet been demonstrated but their existence during turnover cannot be excluded.

Acknowledgements: The support of the Molecular Recognition Initiative of the Science and Engineering Council for the operation of the UEA Centre for Metalloprotein Spectroscopy and Biology is gratefully acknowledged. M.C.C. thanks the SERC-MRI for an earmarked studentship.

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