Biochem. J. (1986) 236, 221–226 (Printed in Great Britain)

Resolution, purification and some properties of the multiple forms of cellobiose quinone dehydrogenase from the white-rot fungus *Sporotrichum pulverulentum*

Fraser F. MORPETH* and Gareth D. JONES

National College of Food Technology (Department of Food Technology), Food Studies Building, University of Reading, Whiteknights, P.O. Box 226, Reading RG6 2AP, U.K.

Four forms of cellobiose quinone dehydrogenase have been purified from the white-rot fungus *Sporotrichum pulverulentum*. The M_r of the enzyme has been estimated by sedimentation equilibrium to be 57800 and by SDS/polyacrylamide-gel to be 60000. These enzymes are clearly monomers. Cellobiose quinone dehydrogenases contain FAD and variable amounts of a green chromophore which we suggest is 6-hydroxy-FAD. The superoxide anion and H_2O_2 are the products of its reaction with oxygen. All of the isoenzymes from any one preparation display similar kinetic parameters. However, these vary between preparations. The only apparent difference between the four separable isoenzymes is their neutral-sugar content.

INTRODUCTION

Cellobiose oxidoreductases have been isolated from a number of fungi capable of cellulose and/or lignin biodegradation (Westermark & Eriksson, 1975; Ayers *et al.*, 1978; Dekker, 1980; Coudray *et al.*, 1982; Morpeth, 1985; Sadana & Patil, 1985). However, the role of these enzymes in biopolymer degradation is not yet clear. In the white-rot fungus *Sporotrichum pulverulentum* (= *Phanerochaete chrysosporium*), resolution of the cellobiose oxidoreductases by ion-exchange chromatography suggests that there are at least three isoenzymes (Morpeth, 1984). One of these is cellobiose oxidase, a flavocytochrome *b*-containing protein (Ayers *et al.*, 1978; Morpeth, 1985). The other two activities seem to be simple flavoproteins (Morpeth, 1984), similar to the cellobiose quinone dehydrogenase purified by Westermark & Eriksson (1975).

The present paper describes the results of experiments on these cellobiose quinone dehydrogenases with a view to understanding their role in ligninocellulose biodegradation.

EXPERIMENTAL

Growth of the organism

Sporotrichum pulverulentum (Novobranova) strain CMI 172 727 obtained from the Commonwealth Mycology Institute was maintained and grown in shaking culture as described previously (Morpeth, 1985).

Separation and purification of the isoenzymes of cellobiose dehydrogenase

The extracellular medium was collected, concentrated and cellobiose oxidoreductase activity separated into three components by chromatography on DEAE-Sephadex as described previously (Morpeth, 1984, 1985). The first activity to be eluted was designated 'isoenzyme 1' and the second 'isoenzyme 2'. A third cellobiose oxidoreductase is the cytochrome *b* flavoprotein, cellobiose oxidase, whose properties have been described elsewhere (Morpeth, 1985; Ayers *et al.*, 1978).

Isoenzyme 1. Peak fractions of cellobiose dehydrogenase isoenzyme 1 were pooled and $(NH_4)_2SO_4$ (285 $g \cdot l^{-1}$) added. The precipitate was collected by centrifugation, redissolved in a minimum volume of 10 mM-Tris/HCl, pH 8.5, and dialysed exhaustively against the same buffer. After dialysis, the solution was applied to a DEAE-cellulose column (1 cm × 30 cm) equilibrated with 10 mM-Tris/HCl, pH 8.5. Isoenzyme 1 was resolved into two active components by this column. One was eluted by 10 mM-Tris/HCl, pH 8.5, containing 50 mM-NaCl, and was designated 'isoenzyme 1a'. The other activity was eluted by 10 mM-Tris/HCl, pH 8.5, containing 250 mM-NaCl, and was designated 'isoenzyme 1b'.

Isoenzyme 1a. Isoenzyme 1a was dialysed exhaustively against 10 mM-potassium phosphate buffer, pH 6, and applied to a concanavalin A–Sepharose column (1.5 cm × 7 cm) equilibrated in the same buffer. The enzyme did not bind to the resin and was eluted by the above buffer. After concentrating to 2 ml, the enzyme was applied to a column of Sephacryl S-200 (2.5 cm × 100 cm) equilibrated with 50 mM-potassium phosphate, pH 6, and eluted with the same buffer.

Isoenzyme 1b. After dialysis against 10 mM-Tris/HCl, pH 8.5, the enzyme was applied to a concanavalin A–Sepharose column (1.5 cm × 7 cm) equilibrated in 10 mM-potassium phosphate buffer, pH 6. This column was washed with the equilibrating buffer, and enzyme activity was eluted by a 1% (w/v) solution of 1-*O*-methyl α-D-glucopyranoside. Active fractions were concentrated to 2 ml by ultrafiltration and applied to, and eluted from, a Sephacryl S-200 gel-filtration column as above.

Isoenzyme 2. The peak fractions of cellobiose dehydrogenase isoenzyme 2 were pooled and precipitated by the addition of $(NH_4)_2SO_4$ (285 $g \cdot l^{-1}$). The precipitate was

* Present address and address for correspondence and reprint requests: I.C.I. Organics Division, P.O. Box no. 42, Hexagon House, Blackley, Manchester M9 3DA, U.K.

collected by centrifugation and redissolved in, and dialysed exhaustively against, 10 mM-Tris/HCl, pH 8.5. After dialysis, the pH of the enzyme solution was lowered to 4.6 with HCl; it was then applied to a CM-Sephadex column (1 cm × 30 cm) equilibrated with 10 mM-sodium acetate buffer, pH 4.6. This column was washed with 50 ml of the equilibrating buffer. Cellobiose quinone dehydrogenase activity was eluted as two separate peaks by washing the column with 10 mM-sodium acetate buffer, pH 4.6, containing 25 mM-NaCl. The first peak of activity was designated 'isoenzyme 2b' and the second 'isoenzyme 2a'. These two isoenzymes were both concentrated to 2 ml by ultrafiltration and applied separately to a Sephacryl S-200 column as described above.

Enzyme assay

Cellobiose quinone dehydrogenase activity was monitored at 25 °C by using the general assay for cellobiose oxidoreductases described previously (Morpeth, 1985). One unit of enzyme activity is defined as the amount of enzyme consuming 1 μmol of cellobiose/min under the above conditions.

Protein concentrations

Protein was estimated by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as a standard. The concentration of bovine serum albumin was determined by using a value of 6.67 for $A^{1\%}_{279}$ (Janatova *et al.*, 1968).

Initial-rate measurements

These were carried out by using a Cary 210 recording spectrophotometer. Since these experiments were for comparative purposes, no attempt was made to exclude oxygen from the assays. Unbiased estimates were obtained by the direct graphical method of Eisenthal & Cornish-Bowden (1974). Initial-rate experiments were carried out in duplicate and were reproducible to within 10% at worst. Extreme care was taken over controlling the pH of the assays, since most experiments were carried out close to the pK of 2,6-dichlorophenol-indophenol.

Amino acid and sugar analysis

Amino acid analyses were performed at the Agricultural Services Unit of this University. Duplicate analyses agreed to within 5%. Tryptophan was determined as described by Pajot (1976), and total carbohydrate was estimated by the method of Dubois *et al.* (1965), with mannose as a standard.

Electrophoresis

Electrophoresis and protein and activity staining of the gels were carried out as described previously (Morpeth, 1985). Isoelectric focusing in polyacrylamide gels was carried out as described by Dickinson & Berrieman (1979).

Analytical ultracentrifugation

This was carried out as described previously (Morpeth, 1985), except that the initial protein concentration of the samples was 0.4 mg/ml. Experiments were carried out on isoenzymes 1b and 2b.

Flavin constituents

The nature of the flavin coenzyme was determined by t.l.c. as described previously (Morpeth, 1985). Resolution of FAD from other flavin chromophores was achieved by using a procedure based on that of Mayhew *et al.* (1974).

Pure cellobiose quinone dehydrogenase (6 ml, A_{450} 0.8), was precipitated at 4 °C with 10% (w/v) trichloroacetic acid. The white denatured apoprotein was discarded by centrifugation and trichloroacetic acid removed by extraction with diethyl ether. The yellow extract was adjusted to neutrality and applied to a column of DEAE-Sephadex equilibrated with 100 mM-potassium phosphate buffer, pH 7. FAD was removed by washing with 200 mM-potassium phosphate, pH 6. A green chromophore was then eluted by washing the column with 100 mM-HCl. This material was collected, freeze-dried and applied to a Bio-Gel P2 column equilibrated with glass-distilled deionized water. The coloured material that was eluted was adjusted to pH 7–8 and applied to a second DEAE-Sephadex column. The chromatographic and elution procedures described above were repeated and the final product was stored in the dark at −80 °C as a freeze-dried precipitate.

RESULTS

The resolution and purification procedure for the isoenzymes of cellobiose quinone dehydrogenase are summarized in Scheme 1. All four pure isoenzymes had similar specific activities, but this varied, from preparation to preparation, between 8.8 and 12.8. The final products are all stable, only slowly losing their activity over several months at 4 °C. Stored in 0.5 ml lots at 3–5 mg·ml^{-1} and −80 °C, all the isoenzymes retained full activity for periods of up to 9 months.

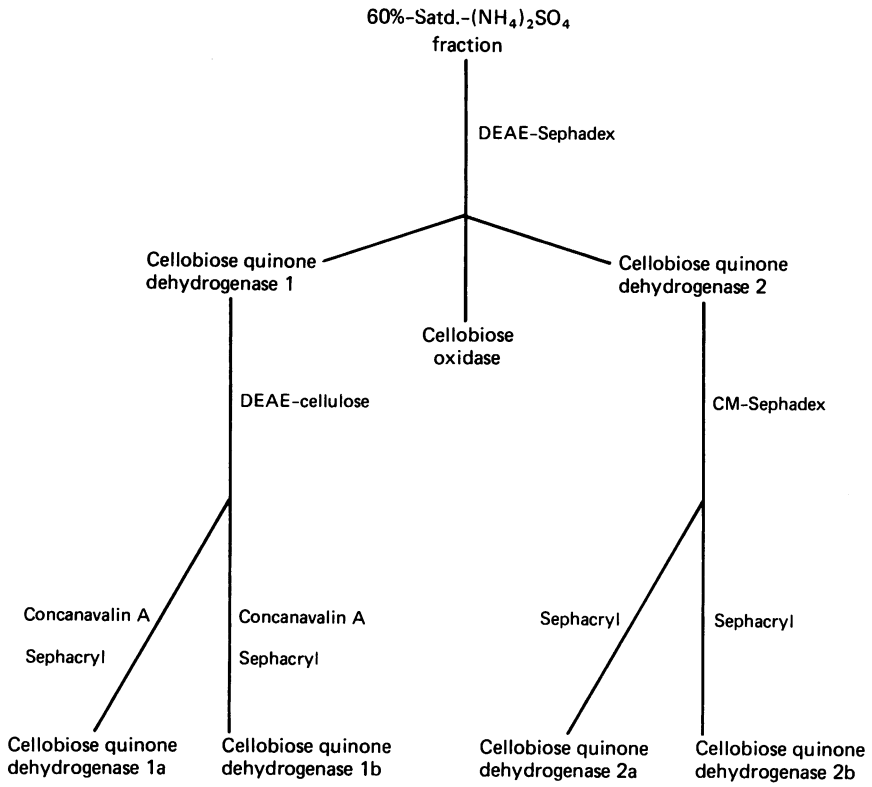
On native gel electrophoresis the isoenzymes all showed one very broad diffuse band, which stained for both activity and protein. On occasions, protein and activity staining of the gel revealed three to five darker staining areas within this broad band. In the presence of SDS, a single broad band was again seen, which corresponds to an M_r of about 60000.

Isoelectric focusing in polyacrylamide gels revealed that all the isoenzymes had two sharp protein and activity bands. In each case there was a major band of pI 6.4 and a minor band of pI 6.2.

In the sedimentation-equilibrium experiments, linear plots were observed over the whole cell. Both isoenzymes examined, 1b and 2a, had an identical partial specific volume, determined by densitometry, of 0.735. The ultracentrifuge runs gave identical results for isoenzymes 1b and 2a, within the limits of error, suggesting an M_r of 57800 ± 2100.

Absorption and fluorescence spectra

The visible absorption spectra of all four cellobiose quinone dehydrogenases vary with different preparations, but are generally similar to that of isoenzyme 1b shown in Fig. 1. This spectrum shows absorption maxima at 457 and 385 nm, with shoulders at 480, 435 and 352 nm. It also has a broad weak absorption band centred at 600 nm. In some preparations the shoulders at 435 and 352 nm appear as distinct peaks (Fig. 2). These spectra differ from the characteristic two-banded absorption spectrum of most simple flavoproteins. They are,



Scheme 1. Purification of the isoenzymes of cellobiose quinone dehydrogenase

A typical preparation starting from 32 litres of culture fluid yielded about 5 mg of isoenzyme 1b and 3 mg of the other isoenzymes.

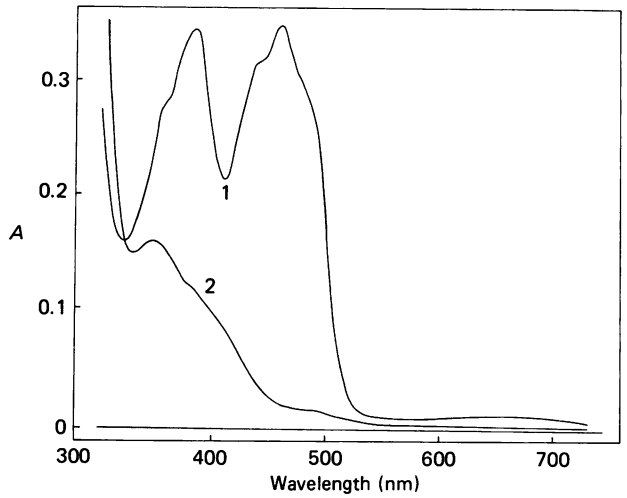


Fig. 1. Absorption spectra of oxidized and reduced cellobiose quinone dehydrogenase 1b

Isoenzyme 1b (26 μM) in 50 mM-potassium phosphate buffer, pH 6, was made anaerobic and the spectrum was recorded at 25 °C (curve 1). Cellobiose (50 μM) was then added from the side arm of the cuvette and the spectrum recorded immediately (curve 2).

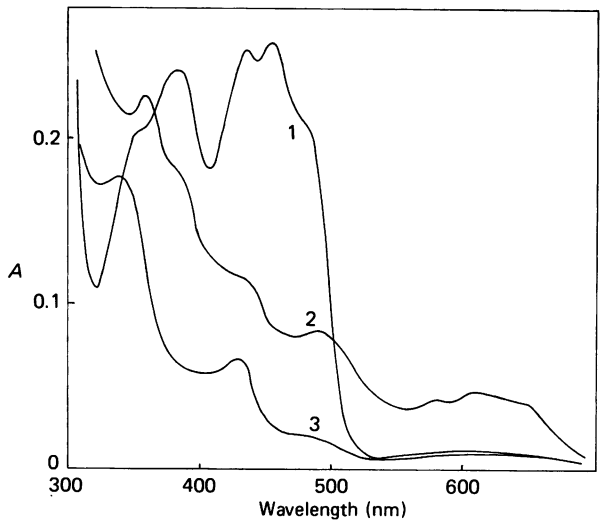


Fig. 2. Absorption spectra of the sulphite adduct and transient semiquinone of cellobiose quinone dehydrogenase 1b

Cellobiose quinone dehydrogenase 1b (17.4 μM) in 50 mM-potassium phosphate buffer, pH 6, was made anaerobic and the spectrum recorded (curve 1). The enzyme was titrated with sodium dithionite until maximum development of the transient semiquinone was seen (curve 2). Curve 3 shows the spectrum obtained after aerobic addition of sodium sulphite to cellobiose quinone dehydrogenase under the same conditions.

however, reminiscent of the visible absorption spectra of pig liver glycollate oxidase (Schuman & Massey, 1971) and an electron-transferring flavoprotein from *Megasphaera elsdenii* (Whitfield & Mayhew, 1974). In addition to their flavin prosthetic groups, these two enzymes also contain small amounts of 6-hydroxy and 8-hydroxy derivatives (Mayhew *et al.*, 1974).

On reduction with cellobiose (50 μM), both the flavin peaks and the absorbance centred at 600 nm are bleached (Fig. 1). No transient flavin semiquinone was seen during

reduction of any of the isoenzymes by cellobiose. The cellobiose quinone dehydrogenases all have an absorbance maximum in the u.v. at 276 nm. The A_{276}/A_{457} ratio in the best preparations was about 12. The FAD in cellobiose quinone dehydrogenases was not fluorescent, in contrast with the proteins, which all had excitation and emission maxima at 280 and 333 nm.

Cellobiose quinone dehydrogenase is rapidly reduced by dithionite. This reduction results in the precipitation of large amounts of the enzyme: 30–50%, as judged by assays of the enzyme activity. In the course of dithionite titrations, a transient blue flavin semiquinone was formed (Fig. 2). Because the enzyme is inactivated, it is possible this semiquinone could be an artefact.

Adding Na_2SO_3 to cellobiose quinone dehydrogenase resulted in a bleaching of the flavin absorbance (Fig. 1). This is due to the reversible formation of a covalent flavin sulphite adduct (Müller & Massey, 1969). Titrations revealed an apparent dissociation constant for sulphite binding of 205 ± 20 μM.

Nature of the reduced oxygen product

In the presence of 30 nmol of cellobiose at 25 °C in 50 mM-potassium phosphate buffer, pH 6, all four isoenzymes of cellobiose quinone dehydrogenase caused a decrease in dissolved oxygen of 27–30 nmol. If catalase was added, 12–14 nmol of oxygen was evolved. Thus H_2O_2 is produced by all the isoenzymes of cellobiose quinone dehydrogenase. When the cellobiose quinone dehydrogenases were incubated aerobically in 50 mM-potassium phosphate buffer, pH 6, with cellobiose (40 μM) and cytochrome *c* (20 μM) at 25 °C, the cytochrome *c* was reduced, depending on the preparation, at between 0.23 to 0.38 mol.s^{-1}.mol of enzyme^{-1}. This was decreased to less than 1% if 5000 units of superoxide dismutase was included in the assay. In the absence of oxygen, cytochrome *c* was not reduced. Thus superoxide and H_2O_2 are produced by cellobiose quinone dehydrogenase.

Identification of the prosthetic groups

The isoenzymes of cellobiose quinone dehydrogenase all contained FAD, as judged by t.l.c. analysis of the prosthetic groups released by 5%-(w/v)-trichloroacetic acid precipitation of the protein. Chromatography of the prosthetic groups on DEAE-Sephadex yielded two major components: FAD and a non-fluorescent green chromophore whose optical spectrum at high and low pH is shown in Fig. 3. These two species could be interconverted with a pK of 7 ± 0.2. The absorbance maxima of the acid and alkali form of this chromophore were 423 nm and 600 nm and 427 nm and 325 nm respectively. This suggests that the green chromophore is 6-hydroxy-FAD (Mayhew *et al.*, 1974).

Carbohydrate content

The isoenzymes of cellobiose quinone dehydrogenase all interact with concanavalin A–Sepharose to different extents. Isoenzyme 1a does not bind, isoenzymes 1b and 2a bind weakly and may be removed by 1% 1-*O*-methyl α-D-glucopyranoside, whereas isoenzyme 2b binds strongly and is only eluted by 10% 1-*O*-methyl α-D-glucopyranoside. Analysis showed that the isoenzymes had the following total neutral sugar content (w/w): 1a, 0.3%; 1b, 0.8%; 2a, 0.9%; 2b, 1.6%.

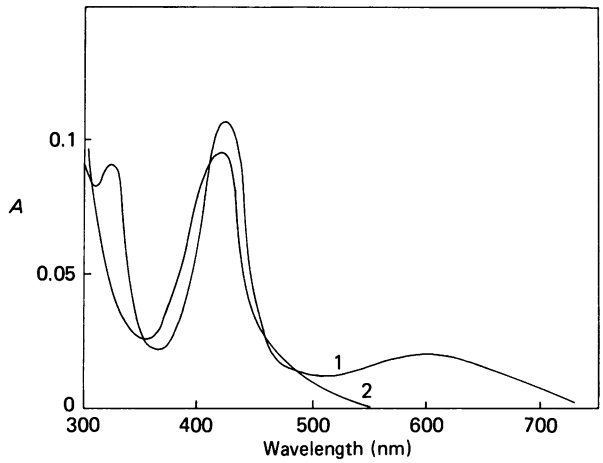


Fig. 3. Effect of pH on the spectrum of the green chromophore

The freeze-dried, purified green chromophore from cellobiose quinone dehydrogenase was redissolved in 10 mM-Tris/HCl, pH 9 (curve 1). Solid citric acid was added in increments and the pH was determined after each addition. The spectrum shown in curve 2 is that at pH 5.1 obtained at the end of the titration.

Amino acid analysis

Within the limits of error the amino acid compositions of 24 h hydrolysates of all four isoenzymes of cellobiose quinone dehydrogenase were the same. The amino acid composition of cellobiose quinone dehydrogenase is shown in Table 1. As with other fungal extracellular enzymes, there is a low content of basic amino acids.

Specificity

The results of assays of the isoenzymes of cellobiose quinone dehydrogenase from one preparation with various substrates and electron acceptors are shown in Table 2. Of the monosaccharides tested, only glucose had any activity. Unlike cellobiose oxidase, crystalline cellulose did not reduce any of the isoenzymes.

Cellobiose gave the lowest K_m and highest $V_{max.}$ of the substrates examined. Benzoquinone gave the highest $V_{max.}$ of the electron acceptors and 2,6-dichlorophenol-indophenol the lowest K_m. Substrate inhibition was seen with 2,6-dichlorophenol-indophenol at concentrations of cellobiose greater than 200 μM. This was not seen with any of the other substrate pairs.

In Table 3 the results of assays of different preparations of cellobiose quinone dehydrogenase 1b are shown. The Michaelis constants for cellobiose and 2,6-dichlorophenol-indophenol vary significantly between preparations. Since cellobiose quinone dehydrogenases contain various amounts of the novel flavin analogue 6-hydroxy-FAD, this is possibly not surprising. However, the changes in the Michaelis constants of the substrates cannot be correlated with changes in the enzyme's spectral properties. Thus it is probable that this variation is due to some other modification of the protein's structure.

DISCUSSION

There are numerous reports in the literature of multiple isoenzymes of various extracellular fungal enzymes (e.g. Eriksson & Pettersson, 1975; McHale & Coughlan, 1981;

Table 1. Amino acid composition of cellobiose quinone dehydrogenase

Each value represents an average of the results obtained for all four isoenzymes after 24 h of hydrolysis. Threonine and serine were estimated by extrapolation to zero time from results with isoenzyme 1b at 24, 48 and 72 h of hydrolysis. The value for isoleucine is that obtained after 72 h of hydrolysis. Cysteine and methionine were determined after formic acid oxidation and tryptophan was estimated by the enzymes' fluorescence in 6 M-guanidinium chloride (Pajot, 1976).

Amino acid	Composition (mol/mol of protein of M_r 57800)
Aspartate	71
Threonine	45
Serine	45
Glutamate	48
Proline	24
Glycine	68
Alanine	67
Valine	42
Methionine	3
Isoleucine	34
Leucine	39
Tryosine	7
Phenylalanine	22
Lysine	15
Histidine	10
Arginine	16
Tryptophan	4
Cysteine	1

Table 2. Apparent Michaelis constants and maximum velocities of various substrates of the isoenzymes of cellobiose quinone dehydrogenase

Values for K_m and $V_{max.}$ were determined by direct linear plots. For the determination of K_m values for sugar substrates, the concentration of 2,6-dichlorophenol-indophenol (DCIP) was held constant at 50 μM. For the K_m values of electron acceptors the concentration of cellobiose was held constant at 200 μM. Maximum velocities were calculated assuming a maximum specific activity of 12.8 units/mg and an M_r of 57800 for cellobiose quinone dehydrogenase. All values are for 25 °C and pH 6 and were determined in 50 mM-potassium phosphate buffer.

Isoenzyme	Varied substrate	K_m (app.) (μM)	$V_{max.}$ (app.) (s^{-1})
	Sugars		
Ia	Cellobiose	46	16
	Lactose	700	11.6
	Maltose	3800	1
	Glucose	400000	1.2
Ib	Cellobiose	42	14.8
	Lactose	680	11.4
	Maltose	3650	1
	Glucose	380000	1.2
IIa	Cellobiose	45	15
	Lactose	740	11.5
IIb	Cellobiose	45	15.4
	Lactose	700	12
	Electron acceptors		
Ia	DCIP	13	15
	Benzoquinone	111	190
Ib	DCIP	15	15
	Benzoquinone	130	21.4
	$K_3Fe(CN)_6$	3100	0.3
IIa	DCIP	14	15.2
	Benzoquinone	102	233
IIb	DCIP	17	15.4
	Benzoquinone	120	21.6

Wood & McCrae, 1979), although the nature of this heterogeneity is seldom clear. Thus the present results are of some interest, since the cellobiose quinone dehydrogenases described above are very similar enzymes, apparently only differing in their neutral-sugar content. The difference in the extent of glycolysation could be generated after excretion, or multiple forms of the enzymes may be released by the fungus.

One difficulty of working with cellobiose quinone dehydrogenases is the variation between preparations in the enzymes' kinetic parameters (Table 3). This is not due to the presence of variable amounts of any other cellobiose/quinone-modifying activity, such as β-galactosidase or laccase. Thus it is probably due to a modification of the enzyme, by either a small proteolytic change or oxidation of one or more amino acids by the high levels of activated oxygen produced by this organism (Forney *et al.*, 1982).

In addition to FAD, cellobiose quinone dehydrogenase also contains variable amounts of a modified flavin, 6-hydroxy-FAD. 6-Hydroxyflavin is an important active-site probe of flavoproteins (Massey & Hemmerich, 1980). We have been unable, so far, to prepare apoprotein which will rebind flavin. Thus it has not been possible to prepare cellobiose quinone dehydrogenase containing only 6-hydroxy-flavin. However, by monitoring the native enzymes' absorbance peaks at 600 and 435 nm, it is possible to study the 6-hydroxy-FAD-containing enzyme. The absorbance at 600 and 435 nm disappears on reduction by substrate (Fig. 1), so the 6-hydroxy-FAD derivative is probably active. Free 6-hydroxy-FAD ionizes at pH 7.1 (Mayhew *et al.*, 1974). Bound to cellobiose quinone dehydrogenase, the 6-hydroxy-FAD ionizes with a pK of about 4, judged by a shift in the 435 nm shoulder to 424 nm and the disappearance of the 600 nm absorbance. Thus cellobiose quinone dehydrogenase stabilizes the anionic benzoquinoid form of 6-hydroxy-FAD.

When considered with some of the other properties of cellobiose quinone dehydrogenase, these results show that it is a very atypical flavoprotein. Cellobiose quinone dehydrogenase behaves as what Massey & Hemmerich (1980) describe as a 'C–N transhydrogenase' in that superoxide is the first-determined oxygen product and it stabilizes an unstable blue neutral radical. However, it has two properties not usually associated with this class of enzymes, in that it strongly stabilizes the benzoquinoid form of 6-hydroxyflavin and forms a sulphite adduct. The precise reasons for these ambiguities await further investigation into the chemical nature of the flavin-binding site.

Lignin biodegradation is thought to be brought about by a novel peroxidase (Tien & Kirk, 1984; Glenn *et al.*, 1983) via a radical-cation mechanism (Kersten *et al.*, 1985; Schoemaker *et al.*, 1985). However, many of the peripheral activities in this process are still poorly

Table 3. Apparent Michaelis constants and maximum velocities of different preparations of cellobiose quinone dehydrogenase isoenzyme 1b

Values for K_m and $V_{max.}$ were determined by direct linear plots. For the determination of K_m values for sugar substrates, the concentration of 2,6-dichlorophenol-indophenol (DCIP) was held constant at 50 μM. For the K_m values of 2,6-dichlorophenol-indophenol, the concentration of cellobiose was held constant at 200 μM. Maximum velocities were calculated by assuming an M_r of 57800. All values were determined in 50 mM-potassium phosphate buffer, pH 6, at 25 °C.

Varied substrate	Preparation	K_m (app.) (μM)	$V_{max.}$ (app.) (s^{-1})
Cellobiose	1	42	14.8
	2	60	16
	3	46	11.6
	4	72	14
	5	30	12
DCIP	1	15	15
	2	15	16
	3	25	11.4
	4	15	15
	5	28	12.5

understood. Quinones are possible intermediates in the lignin breakdown (Buswell *et al.*, 1982; Lundquist & Kristersson, 1985); cellobiose quinone dehydrogenase could be involved in controlling their levels and in providing an important link between carbohydrate and lignin metabolism.

We thank Ms. S. L. Pillinger and Dr. P. H. Stothart for performing the analytical-ultracentrifuge experiments and Mr. C. W. Varnals for technical assistance. This work was supported by a grant from the Science and Engineering Research Council (GR/D.23978) and a University of Reading Research Endowment Fellowship to G.D.J.

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Received 25 October 1985/14 January 1986; accepted 20 January 1986