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Rapid kinetic studies of the reduction of cellobiose oxidase from the white-rot fungus *Sporotrichum pulverulentum* by cellobiose

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The reactions between cellobiose and cellobiose oxidase were investigated by stopped-flow spectrophotometry. Under anaerobic conditions rapid reduction of the associated flavin is followed by slower reduction of cytochrome *b*. The kinetic difference spectra are reported. The rate of flavin reduction depends on the cellobiose concentration (with an apparent second-order rate constant of approx. 10^5 $M^{-1} \cdot s^{-1}$) but reaches a rate limit of approx. 20 s^{-1}. In contrast, the rate of cytochrome *b* reduction decreases at high cellobiose concentrations. Kinetic titrations of the flavin and cytochrome *b* moieties yield the stoichiometries of the separate reactions, i.e. the number of moles of cellobiose needed to fully reduce 1 mole of each redox component. The rate constant for cytochrome *b* reduction, unlike that for flavin reduction, increased with enzyme concentration, prompting the conclusion that any given cytochrome *b* centre is reduced preferentially by flavin groups in different molecules rather than by its partner flavin within the same monomer. These data are discussed in the context of a scheme that rationalizes them and accounts for the overall stoichiometry in which three two-electron donors (cellobiose molecules) reduce two three-electron acceptors (the flavin–cytochrome *b* of cellobiose oxidase).

INTRODUCTION

Cellulose- and lignocellulose-degrading fungi have been identified that enhance their ability to break down these biopolymers by releasing a variety of oxidative enzymes (see Morpeth & Jones, 1986, and references cited therein). Cellobiose oxidoreductases represent one family of such oxidative enzymes whose precise role is as yet undetermined. Since cellobiose quinone dehydrogenase was first identified (Westermark & Eriksson, 1975), its presence has been shown not to be essential for lignin breakdown (Eriksson, 1985), but it is thought to be involved in controlling the concentrations of quinone intermediates involved in this process and linking lignin and carbohydrate metabolism (Lundquist & Kristersson, 1985; Morpeth & Jones, 1986). Cellobiose oxidase, the flavocytochrome *b*-containing member of this family, was first isolated from the white-rot fungus *Sporotrichum pulverulentum* (= *Phanerochaete chrysosporium*) by Ayers *et al.* (1978), and considerable progress was made towards its characterization by Morpeth (1985). It has been suggested that this enzyme helps to enhance the rate of cellulose degradation by removing the inhibitory effect of cellobiose on the activity of the hydrolytic enzymes involved (Ayers *et al.*, 1978), but the enzyme has also been implicated in lignin breakdown directly by virtue of the highly reactive $O_2^{\cdot-}$ radicals that it produces when molecular O_2 is reduced (Forney *et al.*, 1982; Crawford & Crawford, 1984; Morpeth, 1985). However, an increasing body of information suggests that the recently identified 'ligninase', a novel peroxidase, is the major protagonist for enzyme-mediated lignin biodegradation (Tien & Kirk, 1983; Paterson & Lundquist, 1985; Kirk *et al.*, 1986).

The rapid kinetic studies reported in the present paper were undertaken to help clarify the true role of cellobiose oxidase and to aid the understanding of the complex steady-state kinetic behaviour exhibited by this enzyme that was first reported by Morpeth (1985).

MATERIALS AND METHODS

Growth of the organism

Sporotrichum pulverulentum (*Novobranova*) strain C.M.I. 172727 obtained from the Commonwealth Mycology Institute was maintained and grown in shaking culture essentially as described by Morpeth (1985). However, the medium was supplemented with DL-asparagine (1 g/l) and NH_4NO_3 (0.5 g/l).

Purification of cellobiose oxidase

A simplified version of the procedure described by Morpeth (1985) was used to produce pure cellobiose oxidase. Fractions containing cellobiose oxidase collected from the DEAE-Sephadex A-50 column were pooled only if they possessed an $A_{280}^{ox.}/A_{421}^{ox.}$ ratio of 9.4 or less. The resultant enzyme sample was concentrated by ultrafiltration to approx. 10 ml, then passed down the Sephacryl S-200 column without performing the intermediate precipitation step with $(NH_4)_2SO_4$. Fractions from the Sephacryl S-200 column were pooled if they possessed an $A_{280}^{ox.}/A_{421}^{ox.}$ ratio of between 1.85 and 2.1. It was unnecessary to pass such enzyme down the Reactive Red–agarose column described by Morpeth (1985), as in the standard assay it possessed a specific activity typically of between 4.5 and 5 units/mg, which was not improved by this procedure. The supplementation of the growth

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medium with DL-asparagine and NH_4NO_3 resulted in yields of pure enzyme 3–4-fold in excess of the typical yield reported by Morpeth (1985).

Enzyme assay

The activity of cellobiose oxidase was monitored by using the general assay for cellobiose oxidoreductases described by Morpeth (1985) under the conditions stated.

Protein concentration

Protein was determined as described by Morpeth & Jones (1986).

Stopped-flow spectrophotometry

Reactions were carried out in a Durrum–Gibson stopped-flow apparatus with 2 cm observation chamber and dead-time of approx. 3 ms. The apparatus was thoroughly washed with anaerobic buffer before each experimental session commenced.

Absorption spectra were measured and enzyme assays were performed in a Cary 210 recording spectrophotometer. The concentration of stock cellobiose solutions used in the stopped-flow experiments was determined by hydrolysing samples with β-glucosidase (Sigma type X from *Aspergillus niger*) and measuring the glucose formed by using the GOD–Period method supplied by Boehringer Mannheim.

RESULTS

Static spectroscopy

Addition of cellobiose to cellobiose oxidase under anaerobic conditions generated the typical spectrum of the fully reduced protein, characterized by the appearance of distinct α- and β-bands (at 562 nm and 531 nm) of the *b*-type cytochrome component as described by Morpeth (1985). As the protein purification procedure has been modified (see the Materials and methods section), yielding an enzyme with higher specific activity than previously reported, it was necessary to re-determine the absorption coefficient. This parameter was obtained by the standard method of drying samples of solution of known absorbance to constant weight. This procedure gave $\epsilon_{421} \simeq 65200\ M^{-1} \cdot cm^{-1}$, some 12% higher than reported earlier (Morpeth, 1985).

Stopped-flow kinetic studies

On mixing excess cellobiose with cellobiose oxidase under anaerobic conditions in a stopped-flow apparatus, rapid spectral changes were observed consistent with full reduction of the enzyme. The time course was, however, complex, consisting of two phases, the proportions of which were wavelength-dependent. A faster exponential phase was evident around 450 nm, whereas at longer wavelengths, around 560 nm, a slower process with a lag preceding an exponential increase in the absorbance was seen. These processes are illustrated in Fig. 1.

At all wavelengths examined the time course comprised a combination of these phases. The amplitudes of the two phases are plotted as a function of wavelength in Fig. 2 to yield the kinetic difference spectrum of the overall reaction and of the separate processes. It is seen that the total absorbance change at each wavelength is exactly that expected for full reduction of the protein, as identified by the coincidence of the overall kinetic

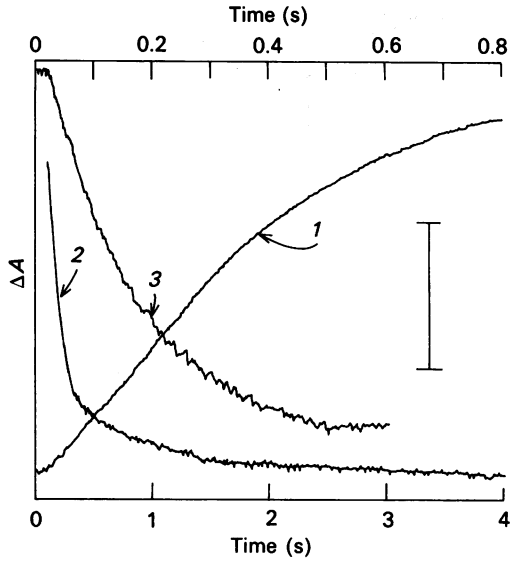

Fig. 1. Time course for the reaction of cellobiose oxidase and cellobiose under anaerobic conditions

After mixing, the concentrations of cellobiose and cellobiose oxidase were 16 μM and 6.0 μM respectively. Trace *1* was recorded at 562 nm and traces *2* and *3* were recorded at 449 nm. The lower time scale refers to traces *1* and *2* and the upper scale refers to trace *3*. The vertical bar represents $\Delta A = 0.05\ A$ for trace *1* and 0.025 *A* for traces *2* and *3*. The reactions were carried out in 50 mM-potassium phosphate buffer, pH 6.0, at 24 °C.

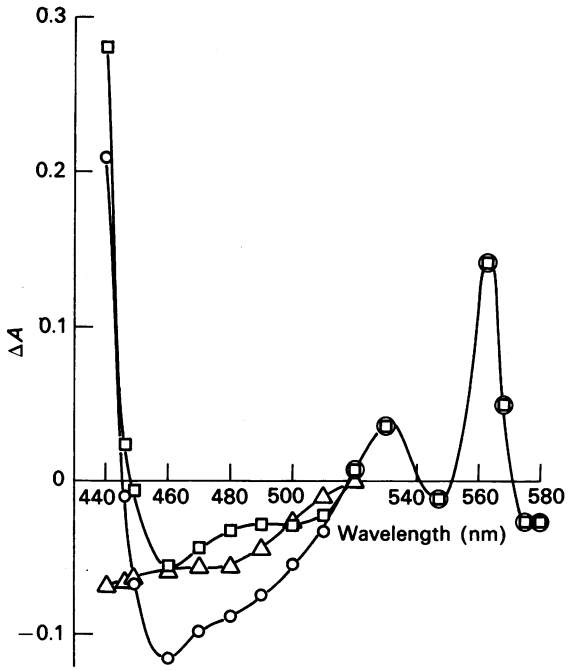

Fig. 2. Kinetic difference spectra for the reduction of cellobiose oxidase by excess cellobiose under anaerobic conditions

△, □ and ○ denote the absorbance change at each wavelength for the fast phase, slow phase and overall reaction respectively. The curve passing through the ○ symbols coincides precisely (in both shape and amplitude) with the static difference spectrum for completely reduced minus oxidized enzyme. After mixing, the concentrations of cellobiose and cellobiose oxidase were 37 μM and 6.1 μM respectively. The reactions were carried out in 50 mM-potassium phosphate buffer, pH 6.0, at 24 °C.

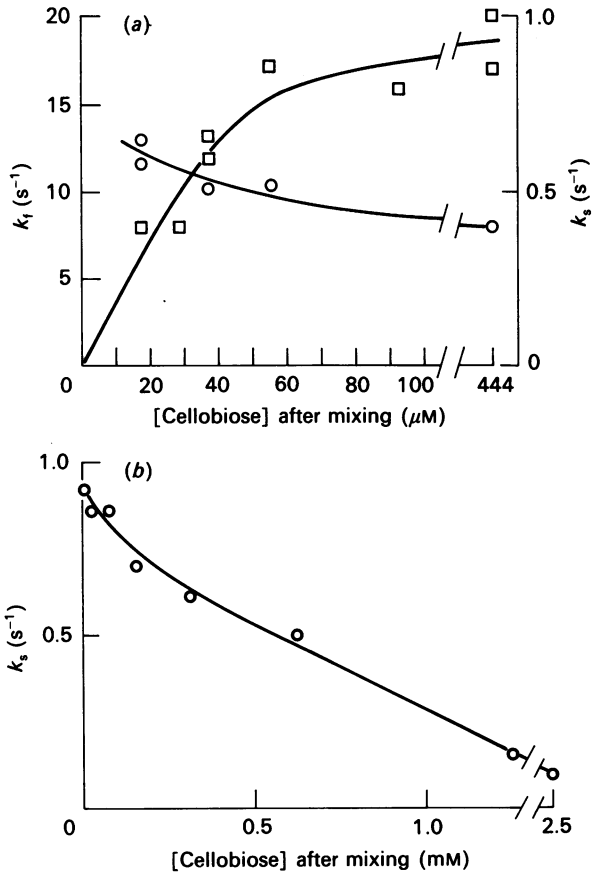

Fig. 3. Cellobiose-concentration-dependence of (*a*) the fast and the slow phases and (*b*) the slow phase of cellobiose oxidase reduction

In (*a*) k_f (□) and k_s (○) refer to the pseudo-first-order rate constants of the fast and the slow phases of reduction, monitored at 449 nm and 562 nm respectively. After mixing, the concentration of cellobiose oxidase was 6.1 μM. Reactions were carried out in 50 mM-potassium phosphate buffer, pH 6.0, at 24 °C. (The more pronounced scatter in the values of k_f reflects a small wavelength error in repeatedly setting the monochrometer to the isosbestic for cytochrome *b*, where $\Delta A/\Delta\lambda$ is large.) In (*b*) k_s (○) refers to the pseudo-first-order rate constant of the slow phase of reduction monitored at 562 nm. After mixing, the concentration of cellobiose oxidase was 6.6 μM. Reactions were carried out in 50 mM-potassium phosphate buffer, pH 6.0, at 22 °C. The k_s values of the slow phases shown in (*a*) and (*b*) were determined on two different enzyme preparations.

difference spectrum and that obtained statically. This indicates that full reduction of the enzyme is being achieved in the stopped-flow spectrophotometer under these conditions and that no fast processes are being missed in the dead-time of the apparatus. The difference spectrum of the slow process, exhibiting sharp peaks at 563 nm and 531 nm, reports the reduction of the cytochrome *b* component. The fast process, leading to bleaching of a broad band around 450 nm, is consistent with the proposal that this process is reporting reduction of the flavin component.

Experiments in which the cellobiose concentration was changed allowed both the kinetics of the reduction of the components and the stoichiometry of the separate reactions to be examined. Fig. 3(*a*) shows the dependence of pseudo-first-order rate constant for flavin reduction on cellobiose concentration. This rate constant increases up to a limiting value of approx. 20 s^{-1}. The exact concentration-dependence at low cellobiose is difficult to obtain, as the substrate concentration becomes close to that of the enzyme and hence first-order kinetic analysis is not applicable. Fig. 3(*a*) also shows the dependence on cellobiose concentration of the rate constant for cytochrome *b* reduction (determined subsequent to the lag illustrated in Fig. 1). Here we see no increase in rate with increase in substrate concentration, but rather a decrease. This is more fully shown in Fig. 3(*b*) from a separate experiment, where it is seen that there is a strong inverse correlation between the rate of cytochrome *b* reduction and the cellobiose concentration. This effect must be correlated with the known substrate inhibition of the enzyme in steady-state turnover (Morpeth, 1985) and must presumably indicate at least two binding sites for the substrate, the active site and an inhibition site.

We interpret Fig. 3(*a*) to mean that electrons rapidly enter the flavin component, probably in a mechanism that involves a pre-equilibrium to form a complex in which the electron transfer is rate-limited, hence explaining the saturation behaviour observed. Electrons are then passed from the flavin to cytochrome *b* (probably through intermolecular electron transfer; see below). The lack of any indication of a second-order process occurring between cellobiose and cytochrome *b* supports this conclusion, as does the time course shown in Fig. 1, which shows that, while the flavin is being reduced (400 ms), the rate of cytochrome *b* reduction is low, i.e. we observe a lag phase. Once the flavin is reduced, the full rate of cytochrome *b* reduction is attained.

Fig. 4(*a*) shows the maximum change in absorbance at 449 nm when cellobiose oxidase is reduced by relatively low concentrations of cellobiose. We interpret changes at this wavelength, isobestic for cytochrome *b* reduction (see Fig. 2), to reflect changes in the redox state of the flavin present in the enzyme. The experimental data illustrated in the left inset to Fig. 4(*a*) indicate that substoichiometric concentrations of cellobiose reduce an equivalent concentration of the flavin, which subsequently becomes largely re-oxidized (see the slower time course) at the rate characteristic of cytochrome *b* reduction (see Fig. 4*b*). When cellobiose concentrations close to stoichiometric with the enzyme redox sites are employed, the absorbance change approaches a limiting value and no re-oxidation of the flavin is evident (see right inset to Fig. 4*a*). This 'kinetic' titration of the flavin by cellobiose allows an estimate of the stoichiometry of the reduction to be made. The data suggest that 1 mol of cellobiose reduces 1 mol of enzyme-bound flavin in the fast kinetic process.

Fig. 4(*b*) shows the 'kinetic' titration of the cytochrome *b* component of cellobiose oxidase. Cytochrome *b* is reduced by cellobiose and the stoichiometry is again close to 1 mol of cytochrome *b* reduced per mol of cellobiose. However, inspection of Fig. 4(*b*) does give some suggestion that the stoichiometry of the reaction may be higher than this and may indeed be non-integral. The highest value to be expected would be 2 mol of cytochrome *b* per mol of cellobiose.

Fig. 5 shows the dependence of the rates of flavin and cytochrome *b* reduction on the concentration of cellobiose oxidase at a constant cellobiose concentration of

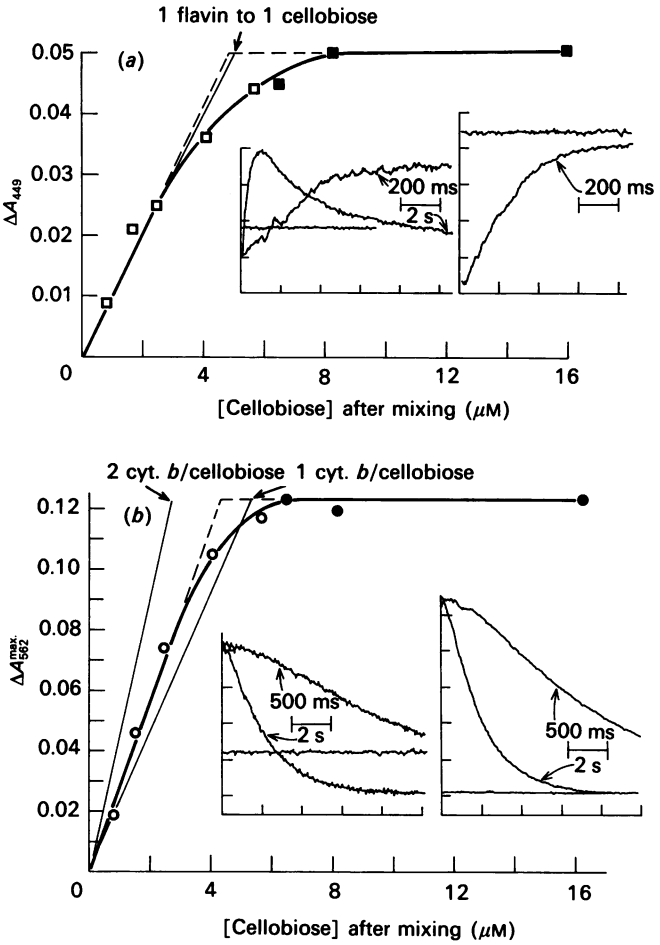

Fig. 4. Cellobiose-concentration-dependence of (*a*) the maximum absorbance change at 449 nm on reduction of flavin in cellobiose oxidase and (*b*) the maximum absorbance change at 562 nm on reduction of cytochrome *b* in cellobiose oxidase

In (*a*) the open symbols refer to experiments performed with 5.3 μM enzyme and the closed symbols with 6.0 μM enzyme. In these latter cases the measured amplitudes were normalized to 5.3 μM enzyme. Left inset: time courses of changes in transmission when 1.6 μM-cellobiose reacts with 5.3 μM-cellobiose oxidase. Right inset: time course of change in transmission when 8.1 μM-cellobiose reacts with 6.0 μM-cellobiose oxidase. Concentrations are given after mixing. All experiments were performed in 50 mM-potassium buffer, pH 6.0, at 24 °C. In (*b*) the open and closed symbols have the same meaning as in (*a*). Left inset: time course of changes in transmission when 1.6 μM-cellobiose reacts with 5.3 μM-cellobiose oxidase; the overshoot is due to partial, very slow, reoxidation of the enzyme due to low-level contamination by O_2. Right inset: time courses of changes in transmission when 8.1 μM-cellobiose reacts with 6.0 μM-cellobiose oxidase. Concentrations are given after mixing. All experiments were performed in 50 mM-potassium phosphate buffer, pH 6.0, at 24 °C.

125 μM. It is evident that flavin reduction (Fig. 5 inset) is independent of the absolute enzyme concentration, as expected from the results given in Fig. 3, and under conditions where the cellobiose is at all times in large excess over the enzyme. The main profile in Fig. 5, however, shows a clear dependence of the rate for

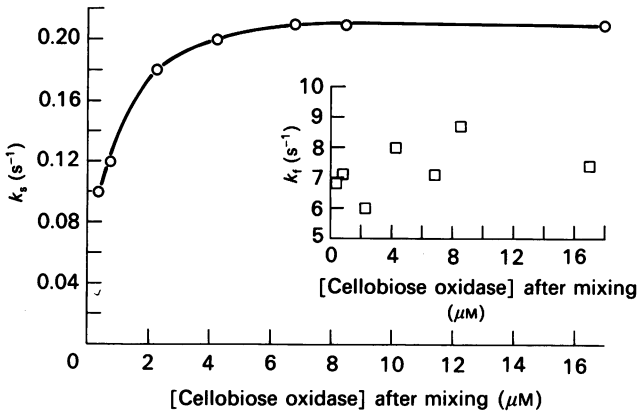

Fig. 5. Enzyme-concentration-dependence of the fast and the slow phases of cellobiose oxidase reduction

k_f (□) and k_s (○) refer to the pseudo-first-order rate constants of the fast and the slow phases of reduction respectively. After mixing, the concentration of cellobiose was 125 μM. Reactions were carried out in 50 mM-potassium phosphate buffer, pH 6.0, at 22 °C.

cytochrome *b* reduction on the enzyme concentration, indicating that this component may be reduced in an intermolecular process that, from the initial slope, is characterized by a second-order rate constant of approx. 10^5 $M^{-1} \cdot s^{-1}$, and that reaches a limiting value of approx. 0.21 s^{-1} under these conditions (moderately high in cellobiose). From the data in Fig. 5 it is not possible to estimate precisely the value of the intercept on the rate-constant axis, which must correspond to the rate constant for the reduction of cytochrome *b* by intramolecular electron transfer from its companion flavin. Whatever the exact value of this constant, it does seem to be very low, suggesting that, in these experiments at least (i.e. anaerobic reduction with no external electron acceptor), intramolecular electron transfer from the fully reduced flavin to cytochrome *b* is not favoured. This may of course be different for the electron transfer from flavin semiquinone to cytochrome *b* (see below).

DISCUSSION

We propose the mechanism shown in Scheme 1 for the anaerobic reduction of cellobiose oxidase by cellobiose, which we believe may accommodate the experimental data presented above. Step (1) depicts the full reduction of flavin by the two-electron donor cellobiose. This process is second-order and limited by the value of k_{et}, as illustrated in Fig. 3. This rapid initial process may be monitored at 449 nm, and the dependence of the amplitude of this signal on cellobiose concentration given in Fig. 4(*a*) is in line with the stoichiometry given by this step. The enzyme-concentration-dependence of cytochrome *b* reduction shown in Fig. 5 is embodied in steps (2)–(4). Step (2) must be very slow compared with the other rates. Steps (3) and (4), being intermolecular, give the enzyme-concentration-dependence. These steps are presumably simplifications and must also include first-order steps within complexes in order to explain the approach to a rate limit in Fig. 5. At high (above stoichiometric) cellobiose concentrations step (3) will predominate, as the species F_ob_o of step (4) will not exist.

$$F_ob_o + C \rightleftharpoons C \cdot F_ob_o \xrightarrow{k_{et}} P \cdot F_rb_o \rightleftharpoons F_rb_o + P \quad (1)$$

$$F_rb_o \rightleftharpoons F^{\bullet}b_r \quad (2)$$

$$F_rb_o + F_rb_o \rightleftharpoons F^{\bullet}b_o + F_rb_r \quad (3)$$

$$F_rb_o + F_ob_o \rightleftharpoons F^{\bullet}b_o + F_ob_r \text{ or } 2F^{\bullet}b_o \quad (4)$$

$$F^{\bullet}b_o \rightleftharpoons F_ob_r \quad (5)$$

$$F_ob_r + C \rightleftharpoons F_rb_r + P \quad (6)$$

Scheme 1.

Abbreviations: F_o, oxidized flavin; $F^{\bullet}$, flavin semiquinone (radical); F_r, fully reduced flavin; b_o, oxidized cytochrome *b*; b_r, reduced cytochrome *b*; C, cellobiose; P, cellobionolactone. k_{et} is the pseudo-first-order rate constant for electron transfer.

Under these circumstances we envisage that the main route by which cytochrome *b* is reduced is by electron transfer from a flavin in a different molecule, leaving a semiquinone paired with oxidized cytochrome *b* as a product. This rapidly undergoes electron redistribution, giving F_ob_r, as shown in step (5). The slow build-up and rapid decay of the semiquinone species results in this species attaining only very low concentrations, thus explaining why we see no spectral evidence for a semiquinone in our experiments (see Fig. 2).

At low cellobiose concentrations the reactions in step (4) may occur, followed rapidly by step (5). This is in keeping with the reoxidation of a fraction of the reduced flavin at the rate of cytochrome *b* reduction seen in titrations at sub-stoichiometric cellobiose concentrations (see left insets to Figs. 4*a* and 4*b*). If the equilibrium in steps (4) and (5) lay completely to the right, we might expect a stoichiometry of reduced cytochrome *b* to cellobiose monitored in our titration of 2:1. If, however, the equilibrium in step (4) lay largely to the left, this value would approach 1:1. Non-integral values between these, as seen in Fig. 4(*b*), may therefore reflect the fact that the position of this equilibrium is poised. This is also borne out by the fact that in Fig. 4(*a*) (left inset) we do not see complete reoxidation of the flavin.

The final step shows the rapid reduction of F_ob_r by cellobiose, thus yielding the overall stoichiometry of the reaction in which three two-electron donors (cellobiose molecules) reduce two three-electron acceptors. This overall stoichiometry is most easily seen to arise from Scheme 1 if we consider the case in which the enzyme concentration is relatively high and the cellobiose in excess. Under these conditions steps (2) and (4) of the Scheme may be ignored. If step (1) is now written in terms of the reaction of 2 molecules of enzyme and 2 molecules of the substrate, we may describe the mechanism in terms of Scheme 2, in which the steps retain the numbers given in Scheme 1.

The interpretation of the kinetic data and the resultant mechanism for reduction of cellobiose oxidase by cellobiose rely on our assumption that the rapid process monitored at 449 nm reflects the change in concentration of fully reduced flavin and not a flavin semiquinone. This seems reasonable (in the absence of independent evidence such as rapid-freeze e.p.r. studies) when one examines the cellobiose-concentration-dependence of the amplitude of absorbance at this wavelength (Fig. 4*a*). The

$$2F_ob_o + 2C \rightleftharpoons 2F_rb_o + 2P \text{ (ignoring intermediate complexes)} \quad (1)$$

$$2F_rb_o \rightleftharpoons F^{\bullet}b_o + F_rb_r \quad (3)$$

$$F^{\bullet}b_o \rightleftharpoons F_ob_r \quad (5)$$

$$F_ob_r + C \rightleftharpoons F_rb_r + P \quad (6)$$

Summation $2F_ob_o + 3C \rightleftharpoons 2F_rb_r + 3P$

Scheme 2.

stoichiometry of 1 cellobiose molecule per flavin molecule required to maximize the amplitude of ΔA indicates that a two-electron reduction of flavin is being reported.

The curious lack of internal electron transfer between flavin and cytochrome *b* within the cellobiose oxidase monomer may reflect the insignificance of this process to the biological function. The very low rate of reduction of cytochrome *b* in our rapid kinetic studies ($k_s < 1\ s^{-1}$) seems to discount the participation of this process during steady-state turnover of the enzyme under the standard assay conditions (enzyme-concentration-independent catalytic-site activity is approx. $6\ s^{-1}$). A similar situation has been reported by Capeillère-Blandin *et al.* (1975) when flavocytochrome b_2 (L-lactate:ferricytochrome *c* oxidoreductase, EC 1.1.2.3) is reduced by L-lactate. In this case, however, intramolecular electron transfer from FMN to cytochrome *b* led to the reduction of the haem prosthetic group. Although the reduction of cytochrome *b* in one enzyme molecule by reduced flavin in another seems an unusual reaction to predominate over the intramolecular exchange, such intermolecular electron-transfer interactions between 'like' redox proteins are documented [e.g. Capeillère-Blandin *et al.* (1975) report that reduced flavocytochrome b_2 is able to reduce inactive deflavocytochrome b_2]. This proposal, however, must be considered tentative at present, as we have scant information as to the kinetic behaviour of the prosthetic groups in this enzyme under experimental conditions that include suitable oxidizing substrates. Preliminary experiments have shown that fully reduced cellobiose oxidase can be oxidized far more rapidly by water-soluble quinones than by molecular O_2 (G. D. Jones & M. T. Wilson, unpublished work).

Clearly, a great deal more investigation will be required to unravel the complexities of the mechanism of action of cellobiose oxidase and to determine the true role that this enzyme plays in lignocellulose degradation. The work reported here represents the initial phase directed towards this goal and has helped to suggest avenues for further studies.

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