Electron transfer from *Phanerochaete chrysosporium* cellobiose oxidase to equine cytochrome *c* and *Pseudomonas aeruginosa* cytochrome *c*-551

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The electron-transfer reactions of cellobiose oxidase (CBO) have been investigated by conventional and by rapid-scan stopped-flow spectroscopy at pH 6.0. Analysis of the absorbance/time/wavelength matrix by Singular Value Decomposition (SVD) confirms earlier studies showing that cellobiose rapidly reduces the flavin group (7.7 s^{-1} ; cellobiose, $100 \mu\text{M}$) which in turn slowly (0.2 s^{-1}) reduces the cytochrome *b* moiety. In the presence of CBO, cellobiose reduces cytochromes *c* in a reaction that does not depend on oxygen or superoxide. The rate limit for this process is independent of the source of the cytochromes *c* and is identical with the rate of cytochrome *b* reduction. Rapid-mixing experiments show that cytochrome *b* may donate electrons very

rapidly to either mammalian cytochrome *c* or bacterial cytochrome *c*-551. The reactions were second-order ($k_c = 1.75 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_{c-551} = 4.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$; pH 6.0, 21°C and $I=0.064$) and strongly ionic-strength (*I*)-dependent: k_c decreasing with *I* and k_{c-551} increasing with *I*. These results suggest the electron-transfer site near cytochrome *b* bears a significant negative charge. Equilibrium gel chromatography confirms that CBO oxidase and positively charged mammalian cytochrome *c* make stable complexes. These results are discussed in terms of a model suggesting an electron-transfer role for cytochrome *b* *in vivo*, possibly connected with radical-mediated cellulose breakdown.

INTRODUCTION

Cellobiose oxidase (CBO) 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 the lignocellulose-degradation system of this micro-organism (Ayers et al., 1978; Morpeth, 1985; Kremer and Wood, 1992a,b; Samejima and Eriksson, 1992). The reaction catalysed by the enzyme is one in which cellobiose is oxidized to cellobionolactone. Although the electron acceptor required for this reaction *in vivo* is not known with certainty, it is known that, *in vitro*, CBO is able to donate electrons to several types of oxidant, for example, molecular oxygen (O_2) and guinones (2,6-dichlorophenol-indophenol; DCIP) (Jones and Wilson, 1988). The rate of reaction with O_2 , however, makes it unlikely that this enzyme functions as an oxidase *in vivo*.

We have previously shown that the cytochrome *b* moiety of cellobiose oxidase is low spin in both redox states and has the unusual His/Met ligand set (Cox et al., 1992). This iron coordination is the same as that in the *c*-type cytochromes, and strongly argues for an electron-transfer rather than a ligand-binding function for this component of the enzyme. To provide greater insight into the electron-transfer properties of the cytochrome *b* moiety and hence throw light on the problem of its role *in vivo*, we have studied its reactions with well-characterized electron acceptors. Here we report the reactions between CBO and either equine cytochrome *c* or *Pseudomonas aeruginosa* cytochrome *c*-551. Although these cytochromes are non-physiological partners of CBO, much information may be gained by understanding the reaction between these two electron-transfer proteins and CBO. For example, these proteins are single-electron acceptors, as opposed to the multi-electron acceptors O_2 and DCIP. In addition, whilst possessing similar redox potentials, the two cytochromes bear opposite net charges at

pH 6.0 (equine cytochrome *c*, pI 10; *P. aeruginosa* cytochrome *c*-551, pI 4.7; thus a net positive and net negative charge is carried with respect to each protein) and can be used to probe the charges at or near the single-electron transfer site of CBO.

MATERIALS AND METHODS

Materials

CBO was prepared as described by Jones and Wilson (1988), and concentrations were measured using the absorption coefficients reported by these authors ($\epsilon_{421} 65200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the oxidized enzyme) *Pseudomonas aeruginosa* cytochrome *c*-551 was prepared by the method of Parr et al. (1976). Horse heart cytochrome *c* (type VI) and bovine erythrocyte superoxide dismutase (SOD) were obtained from Sigma Chemical Co. All other chemicals (analytical grade) were also obtained from Sigma. Bio-Gel P-60 was obtained from Bio-Rad.

Rapid kinetic studies

Rapid kinetic experiments were performed using either a Durrum–Gibson apparatus (dead time 3.3 ms) or an Applied Photophysics Kinetic Spectrometer (dead time 1 ms).

The apparatus for transient spectroscopy is a photodiode array spectrometer (TRACOR TN6500; Tracor Northern, Madison, WI, U.S.A.) adapted to a thermostatically controlled Durrum–Gibson stopped-flow apparatus, as described elsewhere (Bellelli et al., 1990). Data sets were transferred to a MicroVax 3500 computer for analysis, using the software package PRO-Matlab (Mathworks, Natick, MA, U.S.A.). The absorbance/time/wavelength matrix (**A**) may be written as the product of three other matrices, $\mathbf{A} = \mathbf{U} \cdot \mathbf{S} \cdot \mathbf{V}^T$, where **U** and **V** are orthogonal and **S** is a diagonal matrix. The process of finding **U**, **S** and **V** is termed Singular Value Decomposition (SVD). The

columns of the *U* matrix yield base spectra (here difference spectra), a linear combination of which generate any spectrum forming part of the original data set, and the *V* columns are the time courses of these spectral components. The *S* matrix gives the Singular Value (SV) for each *U* and *V* column and assigns the relative contribution which each base spectrum makes to the data set (see Henry and Hofrichter, 1992, for a review). The resulting *V* columns were fitted to the desired kinetic schemes using the program FACSIMILE (Atomic Energy Authority, Harwell, Didcot, Oxon., U.K.) running on an Intel-486-based personal computer.

Equilibrium gel chromatography

Protein-protein interactions were investigated by the method of Hummel and Dreyer (1962). The column eluent was monitored spectrophotometrically at 550 nm ($\epsilon_{550} = 19600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for ferro- minus ferri-cytochrome *c*), an isosbestic point for oxidized and reduced CBO. The amount of cytochrome *c* bound to CBO was determined by planimetry of the trough area relative to an area corresponding to a known amount of cytochrome *c*. Comparison of the amount of bound cytochrome *c* with the total amount of CBO present allows the cytochrome *c* bound/CBO ratio to be determined.

RESULTS AND DISCUSSION

Equilibrium gel filtration

We have used the Hummel and Dreyer (1962) procedure to examine the interactions between equine cytochrome *c* and CBO. Figure 1 shows an elution profile exhibiting peak, plateau and trough regions indicative of complex formation between CBO and cytochrome *c*. Analysis of this profile (see the Materials and methods section) indicates a 2:1 cytochrome *c*-CBO complex formed in 16 mM potassium phosphate buffer, pH 6.0 (*I* 0.0204).

The effects of ionic strength on the stoichiometry of complexes is presented on Figure 1(b), which shows that the ratio of cytochrome *c* to CBO in the complex varies inversely with ionic strength. This suggests that electrostatic interactions between complementary charges on the proteins are involved in complex formation and stabilization (Moore and Pettigrew, 1990). Cytochrome *c* is positively charged at this pH, thus the site of interaction of CBO is likely to be negatively charged. Interpolation on this Figure indicates that a 1:1 complex forms in 19 mM potassium phosphate buffer, pH 6.0 (*I* 0.0242). The inset to Figure 1(a) shows the Brønsted plot of the data. The gradient in this simple formulation is the product of the charges ($z_1 z_2$) responsible for electrostatic interactions in the complex. Although, the assumptions built into this theory are unlikely to be strictly applicable in this complicated situation, it is clear that $z_1 z_2$ has a value (19) which suggests that several charges on each protein contribute to the stabilization of the complex. The negatively charged surface of CBO is capable of tightly binding many molecules of cytochrome *c* at low *I*, and this leads to complications in both the steady state and transient kinetics if experiments are performed at $I < 0.024$, where non-productive binding may occur.

Steady-state reduction of cytochrome *c*

Morpeth (1985), Ayers and Eriksson (1982) and Eriksson et al. (1990) have suggested that CBO produces superoxide anion as a

primary product of O_2 reduction. Morpeth (1985) also reported that although cytochrome *c* was reduced by CBO, this reaction was inhibited by SOD, implying that superoxide anion was the reductant for cytochrome *c*. The concentration of SOD present ($36 \mu\text{M}$) in these experiments was, however, sufficient for it to act as an electron acceptor. Thus, instead of electron transfer to cytochrome *c*, electrons could preferentially reduce SOD (Kremer and Wood, 1992a), seemingly inhibiting cytochrome *c* reduction. In order to investigate further electron transfer to cytochrome *c*, we have studied the reaction of cytochromes *c* with cellobiose in the absence and presence of CBO. Incubation of ferricytochromes *c* or *c*-551 ($10 \mu\text{M}$) with cellobiose ($100 \mu\text{M}$) in 50 mM potassium phosphate, pH 6.0 (*I* 0.06368) at 20°C led to no reduction of the *c*-type cytochromes, i.e. no electron transfer from cellobiose to either of the cytochromes *c*. The addition of CBO ($1 \mu\text{M}$) resulted in the reduction of both *c*-type cytochromes (results not shown). The rate of reduction was independent of the nature of

Figure 1 Equilibrium gel chromatography

(a) CBO and ferricytochrome *c* ($400 \mu\text{l}$ containing $10 \mu\text{M}$ of each protein) in 16 mM potassium phosphate, pH 6.0 (*I* 0.0204) were incubated for 30 min at 20°C and then applied to a Bio-Gel P-60 column ($10 \text{ mm} \times 410 \text{ mm}$) equilibrated with ferricytochrome *c* ($10 \mu\text{M}$) in the same buffer. The eluent was monitored spectrophotometrically, and the cytochrome *c* concentration determined from the oxidized-minus-dithionite-reduced absorbance at 550 nm ($\Delta\epsilon = 19600 \text{ M}^{-1} \cdot \text{cm}^{-1}$), a wavelength isosbestic for cytochrome *b*. (b) The cytochrome *c*/CBO binding ratio was measured at four values of *I*: 0.0127, 0.0204, 0.0255 and 0.0637 in potassium phosphate buffer, pH 6.0. The Brønsted plot is shown in the inset. The arrows indicate the interpolated value of the *I* required for the formation of a 1:1 complex.

Figure 2 Time-resolved difference spectra recorded during the anaerobic reduction of CBO by cellobiose

Spectra (60 in total) were acquired over the time range 5 ms to 60 s (logarithmic scale) in the wavelength region 400–700 nm and are plotted in a three-dimensional frame taking as reference the spectrum of the fully reduced enzyme. An anaerobic solution of CBO (7 μ M) in potassium phosphate buffer, 50 mM, pH 6.0 was mixed with a solution of cellobiose (100 μ M) in the same anaerobic buffer. The temperature was 20 °C.

the cytochrome *c* and consistent with the relatively slow reduction of the *b*-type haem (via flavin) in CBO by cellobiose at this pH (Jones and Wilson, 1988). The α - and β -peaks at 550 and 520 nm respectively, indicative of the reduction of cytochromes *c*, were clearly resolved, both in the absence and presence of SOD, and no detectable difference to the rate of reduction of ferricytochrome *c* was observed. The reduction of cytochrome *c*, under both conditions, leads to the conclusion that superoxide anion played no part in the electron-transfer process between CBO and cytochrome *c*, reduction occurring by more direct means. Furthermore, steady-state assays in which the initial rate of reduction of horse heart cytochrome *c* was measured at low enzyme concentration (0.1 μ M) and over a range of cytochrome *c* concentrations yielded a value of $k_{\text{cat}} = 0.4 \text{ s}^{-1}$ at pH 6.0 ($I/0.05$). This value is very close to that found for cytochrome *b* reduction by cellobiose (via flavin) [see below and Jones and Wilson (1988)] and supports the view that cytochrome *c* reduction is rate-limited by the rate of ferrocycytochrome *b* production and that it is cytochrome *b* which is the electron donor for cytochrome *c*.

At low I (0.01) the value of k_{cat} fell to below 0.05 s^{-1} which we believe reflects, in part, sequestering and immobilization of cytochrome *c* in non-productive complexes on the surface of the enzyme, as indicated by the high binding ratios seen in Figure 1(a) under these conditions.

Rapid-reaction kinetics

(i) Reduction of CBO by cellobiose

Upon mixing CBO with cellobiose anaerobically the enzyme became reduced over the course of approx. 20 s. Figure 2 shows the 60 spectra of 1024 wavelength readings (acquired on a logarithmic timescale from 5 ms to 1 min) plotted in a three-dimensional frame. The spectra are plotted as differential spectra, taking as reference the spectrum of the fully reduced enzyme (at infinite time). Figure 3 shows the SVD analysis of the same experiment and indicates that, to a good approximation, only the first two U and V columns are required to describe the full data

Figure 3 SVD analysis of the time course of CBO reduction

(a) The 60 spectra depicted in Figure 2 were subjected to **SVD** analysis. The first four **U** columns (pseudo-spectra) are shown. The corresponding **S** values were 4.550, 0.400, 0.049 and 0.020. (b) Fits of the **V** columns (amplitude vectors) to Scheme 1. The two significant **V** columns obtained from **SVD** analysis of the experiment shown in Figure 2 were fitted to a sequential two-step kinetic scheme. The symbols denote experimental points, while the continuous lines are the fitted time courses for $k_1 = 7.7 \text{ s}^{-1}$ and $k_2 = 0.2 \text{ s}^{-1}$.

set (the third and fourth **S** values being, respectively, 10% and 5% of the second **S** value, see legend to Figure 3). Therefore, only the first two **V** columns were used for the fitting to a simple kinetic scheme requiring two kinetic transitions. The bimolecular reaction between CBO and cellobiose was treated as a pseudo-first-order process. A simple scheme of this type may be written as follows:

The results of the fit to this mechanism are reported in Figure 3(b). Although an over-simplification of the process [see Jones and Wilson (1988) and Wilson et al. (1990) for more complex formulations], the fit is nevertheless good. More complex kinetic schemes were examined, but improvement in fitting did not justify the significant increase in the degrees of freedom and are

Figure 4 Reconstructed spectrum of the intermediate species in the reduction of CBO

The spectrum of the intermediate (B), as well as the spectra of the initial (A) and final species (C) were obtained from the fit of the V columns, the time dependent amplitudes of the components. The visible region is shown as a 5-fold amplification.

Figure 5 Difference spectra between the three species observed during reduction of CBO by cellobiose

(a) Difference spectrum between the initial species and the intermediate species. (b) Difference spectrum between the intermediate species and final species.

not elaborated further here. The amplitudes obtained from the fit were used to construct the absolute spectra of the three species depicted in the scheme and which are shown in Figure 4. The spectra at $t = 0$ and $t = \infty$ correspond to those of the fully oxidized and fully reduced enzyme respectively, as expected from the scheme. The spectrum of the intermediate shows it to be that of an enzyme species in which the flavin is fully reduced while the cytochrome b remains fully oxidized. Analysis of the difference between the spectra (Figure 5) supports this conclusion, the spectrum in Figure 5(a) is identical, over the wavelength range explored, with that of the flavin domain prepared from cellobiose oxidase by proteolytic cleavage by Henriksson et al. (1991), while the spectrum in Figure 5(b) is typical of that of ferri-minus ferro-cytochrome b . The first step in reduction of the enzyme, which has a fitted rate constant of 7.7 s^{-1} under these conditions, may thus be attributed to the reduction of the flavin, while the second slower step, having a fitted rate constant of 0.2 s^{-1} , represents electron transfer from reduced flavin to oxidized cytochrome b . This low rate constant for cytochrome b reduction is very close to that reported previously by Jones and Wilson (1988) and close to the value given above for the k_{cat} for cytochrome c reduction. This similarity shows that it is the reduction of cytochrome b that

Figure 6 Time course of the electron transfer from cytochrome b to cytochromes c and c -551

CBO ($3.2 \mu\text{M}$ after mixing), fully reduced with cellobiose, was mixed with either ferricytochromes c ($4.5 \mu\text{M}$ after mixing) (a and c) or c -551 ($4.5 \mu\text{M}$ after mixing) (b and d). In (a) and (b), traces were obtained at 550 nm (c -type cytochrome), and in (c) and (d) traces were obtained at 562 nm (cytochrome b). The upper and lower time scales refer to traces A and B respectively. The reactions were performed in anaerobic 50 mM potassium phosphate buffer, pH 6.0 ($f(0.0637)$) at 20.3°C .

is limiting the rate of cytochrome c reduction by cellobiose, and hence direct electron transfer from flavin to cytochrome c may be discounted. In the kinetic studies reported below we can therefore be sure that we are monitoring direct electron transfer from cytochrome b to cytochrome c .

(ii) Reduction of ferricytochromes c by CBO

The redox potential of horse cytochrome c is 260 mV at pH 6.0 and that of cytochrome c -551 is similar (265 mV), while that of the cytochrome b moiety of CBO is reported to be 165 mV at pH 4.0 and 153 mV at pH 5.0 (Kremer and Wood, 1992a). Kinetic experiments were therefore carried out by mixing cellobiose-reduced CBO with ferricytochrome c . The electron-transfer reaction may be written as follows:

where $K = k_{+1}/k_{-1} = \sim 50$ in favour of cytochrome c reduction (assuming $E^0_{\text{cyt},b} = \sim 150 \text{ mV}$ at pH 6.0).

On mixing the reduced enzyme with either of the cytochromes c , rapid transients were observed in the millisecond time range which, in the presence of excess cytochrome c and cellobiose were followed by slower processes (Figure 6). The initial phase (1) corresponded to oxidation of cytochrome b , monitored at 562 nm , and a synchronous reduction of cytochrome c , monitored at 550 nm . This phase occurs in the first 50 ms following mixing with cytochrome c and within 200 ms with cytochrome c -551. Phase (2) reflects the complete reduction of excess cytochrome c ,

Figure 7 Electron transfer from fully reduced CBO to ferricytochrome *c* under aerobic and anaerobic conditions

The second-order plot of electron transfer to ferricytochrome *c* ($Co = 4.5 \mu\text{M}$) from CBO ($Bo = 7 \mu\text{M}$), fully reduced by the addition of excess cellobiose in 50 mM potassium phosphate buffer, pH 6.0 at 25 °C under both anaerobic ($\square$) and aerobic ($\blacksquare$) conditions (mixing reduced CBO with air-equilibrated cytochrome *c*). Here x is the concentration of either ferrocyanochrome *c* or ferricytochrome *b*.

Figure 8 Cytochrome-*c*-concentration-dependence of electron transfer from fully reduced CBO to ferricytochromes *c* or *c*-551

The pseudo-first-order rate constants of the electron transfer from fully reduced CBO ($1.5 \mu\text{M}$), monitored at 550 nm, are plotted as a function of cytochrome concentration (**a**, cytochrome *c*; **b**, cytochrome *c*-551). The reaction took place in 50 mM potassium phosphate buffer, pH 6.0, at 21 °C.

while cytochrome *b* remains largely oxidized and in steady state. On exhaustion of ferricytochrome *c* cytochrome *b* was re-reduced by the excess cellobiose present, as indicated in phase (3) by an increase in absorbance at 562 nm.

Phase (1) conformed closely to a second-order process (quasi-irreversible), as shown in Figure 7 which yields a value for k_{+1} of $5.5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$. This Figure also reports that this rate constant

Figure 9 *I*-dependence of electron transfer from CBO to cytochromes *c* or *c*-551

The pseudo-first-order rate constants of electron transfer from fully reduced CBO ($1.5 \mu\text{M}$) to cytochromes *c* or *c*-551 (both $5 \mu\text{M}$), shown in (**a**) and (**b**) respectively, were measured over a range of I values (0.00558–0.558). The reaction took place in potassium phosphate buffer, pH 6.0, at 21 °C and was monitored at 550 nm.

for the reduction of cytochrome *c* is insensitive to O_2 , thereby eliminating partially reduced oxygen species (e.g. superoxide anion) as intermediates in the reduction pathway.

The second-order nature of the rapid phase (1) was confirmed by determining the pseudo-first-order rate constants for the reaction between CBO and either cytochromes *c* or *c*-551 over a range of cytochrome concentrations. Plots of the pseudo-first-order rate constants versus either cytochrome *c* or cytochrome *c*-551 concentrations are shown in Figures 8(a) and 8(b). Both rates increase with concentration, and no evidence for approach to a rate limit was observed with either protein over the protein concentration range available. The slopes of these plots yielded second-order rate constants of $1.75 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cytochrome *c* and $4.3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ for cytochrome *c*-551 under these conditions. Only the higher concentration ranges for which pseudo-first-order conditions strictly apply were used to calculate these constants.

The electrostatic interactions between CBO and each of the cytochromes was investigated by measuring the *I*-dependence of the electron-transfer rate constants determined at constant protein concentration. These results are given in Figure 9. The electron-transfer rates to both cytochromes are seen to be strongly *I*-dependent, that of cytochrome *c* increasing (Figure 9a), and that of cytochrome *c*-551 decreasing (Figure 9b), with increasing salt concentration. At the highest salt concentrations studied the rate constant for cytochrome *c*-551 reduction begins to exceed that for cytochrome *c* reduction. At very low *I* the electron-transfer kinetic behaviour became complex (results not shown), the electron-transfer rate apparently falling. This complexity we

attribute to the sequestration of cytochrome *c* in non-productive binding to the surface of CBO (see Figure 1).

Conclusions

The results presented above clearly show that the cytochrome *b* moiety of CBO may act as a facile electron-transfer agent, passing electrons rapidly to other haem proteins. The second-order rate constants, particularly for cytochrome *c* at low *I*, where *k* significantly exceeds $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, are similar to the constants observed for true *in vivo* partners [e.g. cytochrome *c*/cytochrome oxidase; $k = \sim 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ at similar values of pH and *I* (Brunori et al., 1988)]. The value of this rate constant is within an order of magnitude of that reported by Northrup et al. (1993) for electron transfer from bovine ferrocycytochrome *b₅* to yeast iso1 ferrocycytochrome *c* ($13.6 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$, *I* = 0.19; *T* = 25 °C). For this protein pair, structural information is available which, together with analysis of the kinetics, indicate that the proteins dock haem crevice to haem crevice, the haem groups being some 1.2 nm (12 Å) apart. A similar structural motif may underlie the rapid ferrocycytochrome *b* to ferrocycytochrome *c* electron-transfer reaction we observe and indicate that the haem edge of the cytochrome *b* moiety of CBO approaches the surface of the protein (cf. cytochrome *b₅*) and is accessible to the bulk solution. Electrostatic interactions between CBO and cytochrome *c* are important in bringing these components together and enhancing the rate constant in a similar manner to that reported by Northrup et al. (1993) for the ferrocyt *b₅*/iso1 ferrocycytochrome *c* system. The *I*-dependence of the reaction with cytochrome *c* is in keeping with this view, as is the lower value of *k* and the opposite *I*-dependence of the reaction with cytochrome *c*-551, which, although also bearing specific positive charges in the vicinity of the haem *c* edge, nevertheless carries a net negative charge. At high *I*, where electrostatic interactions are largely quenched, cytochrome *c*-551 exhibits the more rapid electron transfer with CBO consistent with its higher self-exchange rate for outer-sphere electron transfer (Keller et al., 1976), as the electron-transfer rate constant increases according to the square root of the self-exchange rate constant [see Marcus and Sutin (1985) for a review].

Both the steady-state and transient kinetic data are consistent with the following simple formulation of the flux of electrons through the enzyme from cellobiose to cytochromes (cyt.) *c*.

The **bold** line depicts the enzyme which comprises a flavin- and a haem-bearing domain. The flavin → cytochrome *b* electron-transfer reaction is shown here for simplicity as an intramolecular step; however, intermolecular transfer is also known to occur (Jones and Wilson, 1988). Thus cytochrome *c* is reduced in turnover at the rate at which cytochrome *b* is reduced by the

flavin, which at pH 6.0 is approx. 0.5 s^{-1} , explaining the catalytic-centre activity ('turnover number') for cytochrome *c* exhibited by the enzyme (see above). This slow reduction of cytochrome *c* is, however, not the intrinsic rate at which electrons may pass from cytochrome *b* to cytochrome *c*, as shown above.

Our conclusion that the cytochrome *b*-to-cytochrome *c* transfer is very rapid at pH 6.0 is in contradiction to earlier reports by Samejima et al. (1992). Those authors have shown that, while cytochrome *c* is reduced by CBO, it is a rather slow process, and we conclude that it was the lower rate, limited by cytochrome *b* reduction (0.3 s^{-1}), which was observed by these authors rather than the second-order process we now report.

The facility with which cytochrome *b* enters into electron-transfer reactions is consistent with its unusual ligand set and suggests an *in vivo* redox function for this component of the enzyme. Several authors have now questioned the role of CBO as a true oxidase (Wilson et al., 1990; Kremer and Wood, 1992a; Samejima and Eriksson, 1992), and Kremer and Wood (1992b) have suggested a redox role in which the cytochrome *b* moiety acts to reduce iron complexes within the local environment of the micro-organism. These ferrous complexes are in turn oxidized by H_2O_2 , initiating Fenton chemistry and thus hydroxyl-radical production. It is these radicals which may chemically attack cellulose in the first steps of a mechanism leading to lignocellulose degradation.

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