Reactions of reduced cellobiose oxidase with oxygen

Is cellobiose oxidase primarily an oxidase?

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We report rapid-mixing experiments in which cellobiose oxidase, fully reduced with cellobiose, is allowed to react with excess molecular O₂. Analysis of the progress curves and their comparison with computer simulations suggests that O₂ reacts only with the cytochrome *b*-type haem and with a rate constant of approx. 0.5 s⁻¹. In steady state the cytochrome *b* is partially oxidized, whereas the flavin remains largely reduced. This situation may be contrasted with that when dichloroindophenol is substituted for O₂. Under these conditions the reactions are rapid (millisecond time range), and the redox centres in the enzyme appear to be oxidized simultaneously.

INTRODUCTION

Fungi capable of utilizing the carbohydrates and lignin of plant cell walls as a food source release a variety of extracellular enzymes to accomplish the task. Some species during their primary growth phase, e.g. the white-rot fungus *Sporotrichum pulverulentum* (= *Phanerochaete chrysosporium*), secrete a family of oxidative enzymes in concert with the hydrolytic enzymes that catalyse the depolymerization of cellulose [see Morpeth & Jones (1986) and references cited therein]. Cellobiose oxidoreductases represent one such group of oxidative enzymes whose true role remains an enigma, even though recent research efforts have yielded significant information concerning the composition and catalytic characteristics of the major isoenzyme forms: cellobiose quinone dehydrogenase and cellobiose oxidase (Morpeth, 1985; Morpeth & Jones, 1986; Odier *et al.*, 1987; Jones & Wilson, 1988).

In a previous publication we have shown that, under anaerobic conditions, cellobiose oxidase is reduced by cellobiose in a biphasic manner. The flavin moiety rapidly accepts two electrons in a second-order process (eventually rate-limited at 20 s⁻¹), whereas the cytochrome *b* moiety is reduced more slowly with a complex mechanism involving inter-enzyme electron transfer. Here we report the results of a first investigation of the other 'half-reaction', namely the reaction of the fully reduced enzyme with the oxidants O₂ and 2,6-dichloroindophenol (DCIP). We conclude from the experiments and their comparison with computer simulations that it is the *b*-type cytochrome which primarily reacts (relatively slowly) with O₂, leading to a steady state in which the flavin and *b*-type cytochrome are held at different levels of reduction. This situation is very different when DCIP is the oxidant, under which circumstances both chromophores are simultaneously and rapidly oxidized (*t*_{1/2} ~ 20 ms).

MATERIALS AND METHODS

Proteins and chemicals

Cellobiose oxidase was prepared from *Sporotrichum pulverulentum* (C.M.I. 172727) by the method described by Jones & Wilson (1988). Enzyme concentrations were determined using $\epsilon_{421} = 65\,200\text{ M}^{-1}\text{ cm}^{-1}$ for the oxidized protein.

Concentrations of 2,6-dichloroindophenol (DCIP; Sigma Chemical Co.) were determined by using an ϵ_{600} value of $13\,800\text{ M}^{-1}\text{ cm}^{-1}$ at pH 6.0. Cellobiose (Sigma) concentrations were checked by the method of Jones & Wilson (1988).

Stopped-flow spectrophotometry

Reactions were performed in a Durrum–Gibson stopped-flow apparatus with a 2 cm observation chamber and a dead time of ~ 3 ms.

Computer simulations

Simulations were performed using the program HAVCHM (Chesick, 1988) on a PC microcomputer.

RESULTS AND DISCUSSION

Fig. 1 shows the results of experiments in which fully reduced cellobiose oxidase, in the presence of cellobiose, was mixed with excess O₂, either 250 μM or 1.2 mM. The reactions were monitored at 562 nm, where only the *b*-type cytochrome contributes to the absorption spectrum, and at 449 nm, where the flavin possesses significant absorbance in the oxidized form and which is isosbestic for the *b*-type cytochrome. After mixing, the *b*-type cytochrome was partially oxidized in a relatively rapid reaction (*t*_{1/2} ~ 500 ms) that showed little O₂-concentration-dependence over the concentration range explored. This phase gave way to a steady state, the length and level of which were determined by the cellobiose and O₂ concentration (see Fig. 1*a* for illustrative progress curves). After cellobiose depletion, the *b*-type cytochrome was fully oxidized by excess O₂. Fig. 1(*b*) shows the parallel experiments monitoring the redox state of the flavin. Here no rapid oxidation is seen (or very little), and a steady state is immediately entered which lies at the fully reduced level. Only when cellobiose is depleted does the steady state collapse and the flavin becomes oxidized. The length of the steady state is O₂-concentration-dependent. It is important to note that, at both wavelengths, there is full recovery of the absorbance change expected from the static difference spectrum of the fully reduced minus fully oxidized enzyme, indicating that no rapid process involving either the flavin or the *b*-type cytochrome occurs within the dead time of the apparatus (~ 2 ms).

Abbreviation used: DCIP, 2,6-dichloroindophenol.

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Fig. 1. Transmission time course for the oxidation of reduced cellobiose oxidase by molecular O_2

Cellobiose oxidase, fully reduced by excess cellobiose, was mixed with oxygenated buffer. In (a) traces were obtained at 562 nm (*b*-type cytochrome) and in (b) traces were obtained at 449 nm (flavin). The substrate concentrations, after mixing, were: traces a and a', 12 μM -cellobiose, 600 μM - O_2 ; traces b and b', 40 μM -cellobiose, 600 μM - O_2 ; traces c and c', 40 μM -cellobiose, 125 μM - O_2 . After mixing, the concentration of cellobiose oxidase was 6 μM . In (b) (cytochrome *b*) the initial rapid oxidation occupies the first 1–5 s (depending on cellobiose and O_2 concentrations) and the steady state lasts 20 s (b) or 60 s (c). In (b) (flavin) no rapid phase was observed and the steady-state phase lasted either approx. 15 s (b') or 50 s (c'). The reactions took place in 50 mM-potassium phosphate buffer, pH 6.0, at 21 $^{\circ}C$.

Previously we have shown that, under these conditions, the flavin is rapidly reduced by cellobiose ($k = 10^5 M^{-1} \cdot s^{-1}$ rate-limited at $\sim 20 s^{-1}$) and that, at this protein concentration, the *b*-type cytochrome is reduced at approx. $0.5 s^{-1}$ by flavin. Taking these results with the finding that we have full absorbance recovery and that the *b*-type cytochrome undergoes the more rapid oxidation of the two redox components (Fig. 1), we propose that it is this *b*-type cytochrome which reacts with O_2 and that the flavin is subsequently oxidized by electron transfer between the two sites. This mechanism may bear comparison with that of neutrophil NADPH oxidase, an enzyme also containing a flavin and a *b*-type cytochrome, the latter reacting with O_2 to produce superoxide anion ($O_2^{\cdot -}$) (Ellis *et al.*, 1989).

This proposed mechanism is consistent with the reduction pathway of the enzyme as shown in the computer simulation (Fig. 2). The mechanism used for simulation is based on a modified form of that proposed by Jones & Wilson (1988) in which, for the purpose of illustration, the reverse of some reactions is omitted. Although this changes the details of the simulation, it does not change the general pattern, as the mechanisms terminates with an irreversible oxidation of cytochrome *b* by O_2 , and this eventually pulls all equilibria to the right.

Fig. 2. Computer simulation of the reaction of cellobiose oxidase with cellobiose and excess O_2

The Figure shows a comparison of actual and simulated stopped-flow experiments monitored at 449 nm (a) and 562 nm (b) (2 cm-pathlength cell), mixing cellobiose-reduced cellobiose oxidase with excess O_2 . Concentrations were: cellobiose oxidase, 6 μM ; cellobiose 40 μM . The smooth lines correspond to the simulations. The values of k_1 , k_2 and k_4 are $10^5 M^{-1} \cdot s^{-1}$, $20 s^{-1}$ and $0.5 s^{-1}$ respectively. The absorption coefficients (ϵ) at 449 nm are: Fobo, $12400 M^{-1} \cdot cm^{-1}$; Fobr, $12400 M^{-1} \cdot cm^{-1}$; Frbo, $6800 M^{-1} \cdot cm^{-1}$; Frbr, $6800 M^{-1} \cdot cm^{-1}$; and at 562 nm: Fobo, $3900 M^{-1} \cdot cm^{-1}$; Fobr, $14460 M^{-1} \cdot cm^{-1}$; Frbo, $3900 M^{-1} \cdot cm^{-1}$; Frbr, $14460 M^{-1} \cdot cm^{-1}$ [rate constants and ϵ values are from Jones & Wilson (1988)]. The fit was obtained taking $k_3 = 10^7 M^{-1} \cdot s^{-1}$, $k_5 = 0.5 s^{-1}$, $\epsilon_{449}^x = 7500 M^{-1} \cdot cm^{-1}$ and $\epsilon_{562}^x = 11250 M^{-1} \cdot cm^{-1}$ (per mol of enzyme). The fit did not depend critically on the value of k_3 , provided it was rapid compared with k_4 , and thus ensured the species X was substantially populated ($\sim 40\%$ of the enzyme) during turnover.

Oxidation by O_2 in a pseudo-first-order process)

Where F and b correspond to the flavin and cytochrome *b* moieties of the enzyme and the suffixes o and r refer to their oxidized and reduced states. C and P refer to cellobiose and cellobionolactone.

The species X denotes a large family of intermediates, populated during steady state, which, for the purposes of this simple simulation, are considered as a single component. The parameters which were varied to obtain this simulation were the value of the rate constant for the reaction of cytochrome *b* with oxygen (k_5) and the (average of the time varying) absorption coefficient of the species X.

Although the broad features of the experimental progress curves are reproduced, it is clear that the fit is only approximate with the values of the rate constants chosen; nevertheless, the main conclusion that cytochrome *b* is the first component to be oxidized seems well founded.

In contrast with the reactions with O_2 , those with DCIP are more rapid, and the two redox sites in the enzyme are oxidized contemporaneously. This is shown in Fig. 3, where the results of mixing reduced cellobiose oxidase with excess DCIP under

Fig. 3. Time course for the anaerobic oxidation of reduced cellobiose oxidase by DCIP

After mixing, the concentrations of cellobiose oxidase, cellobiose and DCIP were $6\ \mu\text{M}$, $12\ \mu\text{M}$ and $21\ \mu\text{M}$ respectively. The total absorbance changes at each wavelength were: 449 nm, 0.028 A ; 562 nm, 0.29 A ; 600 nm, 0.18 A (other conditions as Fig. 1).

anaerobic conditions are given. At all wavelengths we observed a fast process with the same time constant (under these conditions $t_{1/2} \sim 25\ \text{ms}$). At 600 nm this major process leads to an increase in transmission (decrease in absorbance), as expected for the reduction of DCIP which contributes $\sim 95\%$ of the absorbance change at this wavelength. At 562 nm, where the b -type haem

and DCIP make comparable contributions, we see transmission changes the magnitude of which are consistent with the oxidation of $\sim 85\%$ of the b -type haem present ($5.1\ \mu\text{M}$) and reduction of $\sim 6.0\ \mu\text{M}$ -DCIP. At 449 nm a decrease in transmission (increase in absorbance) was seen, consistent with flavin oxidation. Thus, with organic quinone oxidants, the reactions of the enzyme are much more rapid than is the case for O_2 , and the haem and flavin redox centres undergo simultaneous electron-transfer reactions. These results lead us to question whether, *in vivo*, cellobiose oxidase uses O_2 as an oxidant or whether the 'true' substrate(s) is (are) quinone(s) produced from the breakdown of lignin. This depends, of course, on the requirement for O_2^- (and H_2O_2) in the overall breakdown of cellulose and/or lignin and the role these O_2 species may play as a bacteriocidal agent in the natural environment that the white-rot fungus inhabits.

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