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Cellobiose oxidase from *Phanerochaete chrysosporium*

Stopped-flow spectrophotometric analysis of pH-dependent reduction

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Cellobiose oxidase (CBO) from *Phanerochaete chrysosporium* can utilize dichlorphenol–indophenol (Cl_2Ind) and cytochrome *c* as effective electron acceptors for the oxidation of cellobiose. However, the pH dependencies of activity for these electron acceptors are significantly different. Both compounds act as effective electron acceptors at pH 4.2, whereas only dichlorophenol–indophenol is active at pH 5.9. To explain this discrepancy, the pH dependencies of the reduction rates of FAD and heme, respectively, in CBO by cellobiose have been investigated by stopped-flow spectrophotometry. Both FAD and heme are reduced with a high rate constant at pH 4.2. In contrast, at pH 5.9, only FAD reduction is fast, while the reduction of the heme is extremely slow. As a conclusion, the reduction of cytochrome *c* by CBO is dependent on heme, which functions at a lower pH range compared to reduction of FAD.

Cellobiose oxidase; Cytochrome *c*; Dichlorophenol–indophenol; Stopped-flow spectrophotometry; *Phanerochaete chrysosporium*

1. INTRODUCTION

The white-rot fungus, *Phanerochaete chrysosporium* (*Sporotrichum pulverulentum*), produces two different cellobiose-oxidizing enzymes in cellulolytic cultures [1–4]. The first enzyme, cellobiose:quinone oxidoreductase (CBQ) (EC 1.1.5.1), contains FAD as the prosthetic group. The second enzyme, cellobiose oxidase (CBO), has both FAD and b-type heme as prosthetic groups.

In a previous report [5], the catalytic properties of these enzymes were extensively compared. Both CBQ and CBO can effectively reduce dichlorophenol–indophenol, as well as quinones, in the presence of cellobiose. Furthermore, both enzymes also have radical-reducing activities. On the other hand, atmospheric oxygen is a poor electron acceptor for both enzymes. Therefore, CBQ and CBO cannot be distinguished by these catalytic properties. However, CBO is able to utilize cytochrome *c* as an effective electron acceptor, while CBQ cannot. Cytochrome *c* reduction can, therefore, be used as a way to distinguish between CBQ and CBO. Considering the specificity and high activity of CBO for cytochrome *c*, we suggested that cellobiose:cytochrome *c* oxidoreductase might be a more suitable nomenclature than cellobiose oxidase for this enzyme [5].

The reduction of FAD in the two enzymes is considered to couple directly with the oxidation of cellobiose to cellobiono-δ-lactone, because FAD is the only prosthetic group in CBQ [1,4]. Furthermore, the FAD domain alone, derived by proteolysis of CBO with papain, can be reduced by cellobiose [6]. Further electron transfer occurs from the reduced FAD to an appropriate acceptor, including the heme in CBO. In our previous report [5], it was demonstrated that the pH dependence of the reduction rate of these enzymes is significantly different for reduction of dichlorophenol–indophenol and cytochrome *c*. The specificity for cytochrome *c* reduction by CBO suggested that the heme component in CBO was required for this reduction. In order to gain further insight into the mechanism of dichlorophenol–indophenol and cytochrome *c* reduction by CBO, we have investigated the pH dependence of reduction of FAD and heme in CBO by stopped-flow spectrophotometry.

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2. MATERIALS AND METHODS

2.1. *Preparation of cellobiose oxidase (CBO)*

Phanerochaete chrysosporium, strain K-3, was grown in a fermenter on modified Norkans medium [2], containing 2% crystalline cellulose (w/v) as carbon source. The culture was harvested after 4 days of incubation at 37°C and the extracellular solution (the crude enzyme solution) was obtained by filtration on a glass microfiber filter.

Crude enzyme solution, 800 ml, containing 10.4 g protein, was dialyzed against 20 mM sodium acetate, pH 5.0, and applied to a DEAE-Sephadex A-50 column (4.2 × 10 cm). The column was then washed with 2 l of 20 mM sodium acetate, pH 5.0. The CBO-containing fraction, assayed for dichlorophenol–indophenol (Cl_2Ind) and cytochrome *c* reducing activities in the presence of cellobiose, was eluted with 500 ml of the same buffer containing 400 mM NaCl. Active fractions were collected, concentrated using an Amicon PM-10 system, dialyzed against 20 mM sodium phosphate, pH 7.8, then applied to a DEAE-Toyopearl 650M (Tosoh Co., Tokyo, Japan) column (4.5 × 12 cm). The column was washed with 200 ml of 20 mM sodium

phosphate, pH 7.8, and CBO eluted with the same buffer applying a gradient over 0–100 mM KCl in 1.2 l. Active fractions were collected, dialyzed against 10 mM sodium acetate, pH 5.9, and applied to a QAE-Toyopearl 550C column (3.0 × 13 cm). CBO was eluted with a linear gradient of 10–500 mM sodium acetate in 1 l, and 23 mg of CBO was obtained. Purified CBO gave a single protein band on SDS-PAGE analysis (Phast system, Pharmacia-LKB) in the appropriate media according to the manufacturer's manual. The absorption maxima of the purified CBO were observed at 278 nm (110 $mM^{-1}\cdot cm^{-1}$) and 421 nm (61.6 $mM^{-1}\cdot cm^{-1}$), and A_{421}/A_{278} was 0.56.

2.2. *Enzyme assay*

Enzyme assays, using cytochrome *c* and Cl_2Ind as electron acceptors, were carried out at 30°C in 20 mM sodium acetate containing 500 μM cellobiose as an electron donor, pH 4.2 or 5.9, according to our previous report [5]. The absorption coefficients (ε_{600}) for Cl_2Ind used for the calculation of the rate constants are 2.0 (pH 4.2) and 9.3 $mM^{-1}\cdot cm^{-1}$ (pH 5.9), respectively.

2.3. *Stopped-flow spectrophotometry*

The stopped-flow measurements of CBO reduction by cellobiose were performed using a Kinetics Instruments (Bethesda, MD) stopped-flow mixer at 30°C in 20 mM sodium acetate, pH 4.2 or 5.9. The concentrations of CBO and cellobiose after mixing were 8.9 and 500 μM, respectively. Furthermore, the stopped-flow measurement of cytochrome *c* reduction by pre-reduced CBO was conducted by mixing an equal volume of 7 μM CBO, pre-reduced with 500 μM cellobiose, and 10 μM cytochrome *c* (type V-A, c-2037, Sigma) in the same buffer system described above. The rate constant for cytochrome *c* reduction was estimated by the absorbance changes of the α-band of cytochrome *c* at 550 nm. The experimental conditions for rapid-scanning spectrophotometry and for single-wavelength measurements were as previously described [7,8]. Difference spectra were calculated by subtraction of the first spectrum, obtained within 20 ms after mixing, from subsequent spectra.

3. RESULTS

3.1. *Dichlorophenol–indophenol and cytochrome* c *reduction by cellobiose oxidase*

The reduction rates of dichlorophenol–indophenol (Cl_2Ind) and cytochrome *c* by cellobiose oxidase (CBO) were measured at pH 4.2 and 5.9, respectively. The rate constants are presented in Table I. Cl_2Ind was reduced effectively by CBO at both pH values, whereas active cytochrome *c* reduction was observed only at pH 4.2.

3.2. *Static spectra of cellobiose oxidase*

The absorption spectra of native (oxidized) and reduced CBO in 20 mM sodium acetate at pH 4.2 are shown in Fig. 1. The strong Soret-band of the oxidized CBO was observed at 421 nm (61.6 $mM^{-1}\cdot cm^{-1}$). CBO was completely reduced by addition of excess cellobiose.

Table I

Rate constants of dichlorophenol-indophenol (Cl_2Ind) and cytochrome *c* reduction by CBO in 20 mM sodium acetate

	Rate constant (s^{-1})	
	pH 4.2	pH 5.9
Cl_2Ind	13.9	7.1
Cytochrome *c*	13.8	0.3

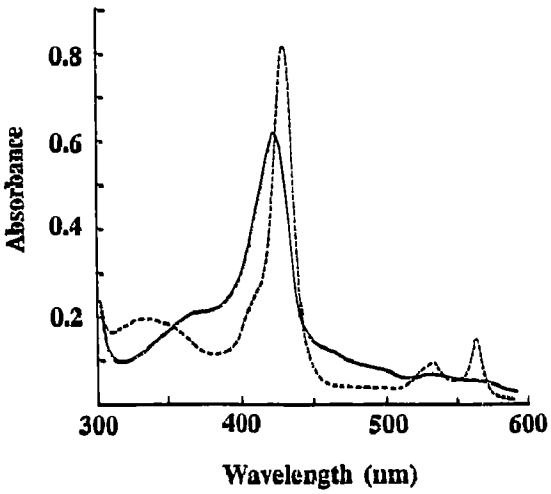

Fig. 1. Static absorption spectra of the oxidized (solid line) and reduced form (dashed line) of cellobiose oxidase (10 μM) in 20 mM sodium acetate, pH 4.2.

The absorption maxima of the reduced CBO were 330 (19.6 $mM^{-1}\cdot cm^{-1}$), 430 (81.7 $mM^{-1}\cdot cm^{-1}$), 532 (9.3 $mM^{-1}\cdot cm^{-1}$), and 563 nm (14.4 $mM^{-1}\cdot cm^{-1}$), respectively. These absorptions are related to b-type heme in CBO. FAD, also contained in CBO [3], must have absorption maxima between 350–500 nm. However, the strong heme absorption obscured the observation of the FAD absorption. The distinct isosbestic points between the oxidized and reduced CBO were observed at 350, 423, 446, 515 and 571 nm, respectively. The spectroscopic features of CBO at pH 5.9 were nearly identical to those at pH 4.2.

3.3. *Rapid-scanning stopped-flow spectra*

CBO and cellobiose were mixed in the stopped-flow instrument, and the rapid-scanning spectra were measured between 350–600 nm. As shown in Fig. 2a, the difference spectra at pH 4.2 indicated that the heme, as well as the FAD, in CBO is reduced very rapidly. The reduction was almost complete within 160 ms. Four distinct isosbestic points were observed, at exactly the same wavelengths as those in the static spectra of CBO. These isosbestic points indicate that accumulation of intermediates could not be observed. In contrast, the difference spectra at pH 5.9 (Fig. 3a) indicated that the CBO reduction is composed of two different stages. In the first stage, the decrease of absorption between 350–500 nm is observed during 100 ms. These changes are mainly caused by the reduction of FAD, and only very slight reduction of the heme is observed with an increase of absorption at 430 and 562 nm. The second stage, however, proceeds very slowly, and it takes more than 10 s to reach the complete reduction. The spectroscopic changes in this stage are caused by the heme reduction, and the isosbestic points related to the heme reduction were observed at 422, 448, 515 and 571 nm, respectively.

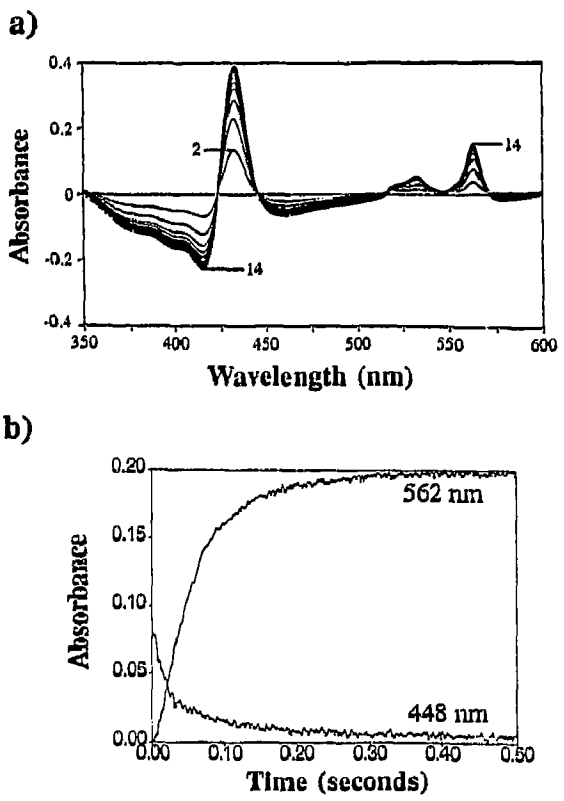

Fig. 2. Time-course of the reduction of cellobiose oxidase (8.9 μM) by 500 μM cellobiose in 20 mM sodium acetate at pH 4.2. (a) Rapid-scanning difference spectra scans were collected at 0.04 s (curve 2), 0.06 s (curve 3), 0.08 s (curve 4), 0.10 s (curve 5), 0.12 s (curve 6), 0.14 s (curve 7), 0.16 s (curve 8), and 0.28 s (curve 14). (b) Absorption changes at 562 and 448 nm.

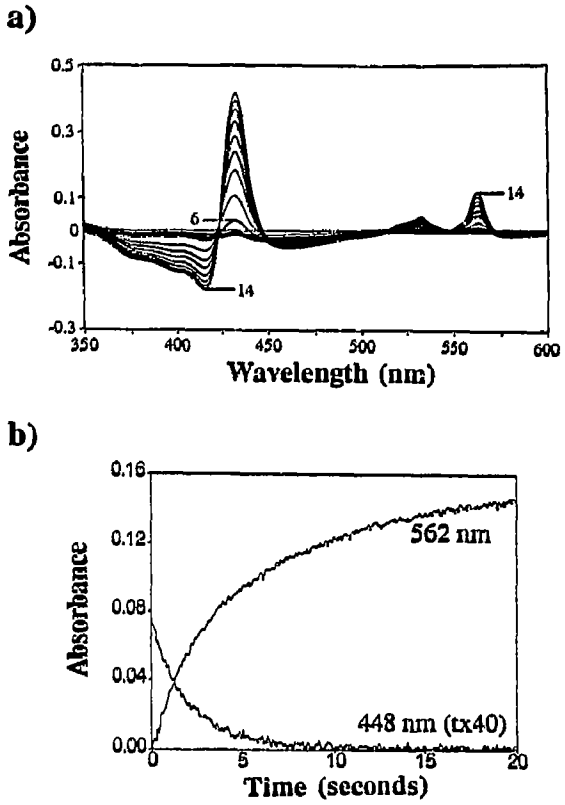

Fig. 3. Time-course of the reduction of cellobiose oxidase (8.9 μM) by 500 μM cellobiose in 20 mM sodium acetate at pH 5.9. (a) Rapid-scanning difference spectra scans were collected at 0.04 s (curve 2), 0.06 s (curve 3), 0.08 s (curve 4), 0.10 s (curve 5), 0.42 s (curve 6), 0.82 s (curve 7), 1.42 s (curve 8), 2.02 s (curve 9), 2.62 s (curve 10), 3.42 s (curve 11), 4.22 s (curve 12), 5.02 s (curve 13), and 5.82 s (curve 14). (b) Absorption changes at 562 and 448 nm. (For the data at 448 nm the time axis has been multiplied 40 times).

3.4. *Rate constants for the reduction of FAD and heme in CBO*

The absorption changes at 448 and 562 nm are considered to be specific for FAD and heme reduction, respectively. The reduction of FAD and heme was, therefore, investigated at these wavelengths by the stopped-flow technique, and the rate constants of FAD and heme reduction were calculated by fitting the progress curves presented in Figs. 2b and 3b. The calculations of the rate constants were made using SIFIT program from On-Line Instruments (Jefferson, GA, USA).

Both FAD and heme reduction appear as pseudo first-order reactions. However, in all cases, these reductions are bi-phasic, composed of two exponential relaxations, suggesting the reduction mechanism is complex. Rate constants for each phase are listed in Table II. The rate constants for FAD reduction are very similar at pH 4.2 and 5.9. However, the rate constant for heme reduction at pH 5.9 is extremely low compared with the rate constant at pH 4.2.

3.5. *Rate constants for cytochrome* c *reduction by the pre-reduced CBO*

The reduction of cytochrome *c* by the pre-reduced CBO was examined at pH 4.2 and 5.9. The resulting rate constants (s^{-1}) for cytochrome *c* reduction are 3.95 at pH 4.2 and 0.05 at pH 5.9, respectively. This result suggests that cytochrome *c* is reduced effectively by the heme in CBO only at pH 4.2.

Table II

Rate constants of FAD and heme reduction in CBO by cellobiose in 20 mM sodium acetate

		Rate constant (s^{-1})	
		pH 4.2	pH 5.9
FAD	Phase I	57.9	45.0
	Phase II	8.6	16.7
Heme	Phase I	23.3	0.60
	Phase II	7.0	0.12

4. DISCUSSION

The difference between CBO and CBQ is that CBO has a heme prosthetic group in addition to the FAD group in CBQ [2,3]. Cytochrome *c* is an electron acceptor specific for CBO [5]. It, therefore, seemed reasonable to assume that the heme in CBO must be responsible for the cytochrome *c* reduction. In contrast, dichlorophenol–indophenol (Cl_2Ind) is an electron acceptor for both CBO and CBQ [3–5], indicating that the heme component in CBO is not necessary for Cl_2Ind reduction.

The pH dependencies of Cl_2Ind and cytochrome *c* reduction by CBO are significantly different. Both Cl_2Ind and cytochrome *c* can be reduced effectively at pH 4.2. However, at pH 5.9, only Cl_2Ind is efficiently reduced. Based on these observations, we investigated the pH dependence of FAD and heme reduction in CBO by stopped-flow spectrophotometry. As shown in Fig. 2, both FAD and heme are reduced rapidly at pH 4.2, whereas at pH 5.9, only FAD is reduced rapidly, followed by a very slow reduction of heme (Fig. 3). Our observation at pH 5.9 is very similar to that reported by Jones and Wilson for pH 6.0 [9]. Therefore, one electron is transferred effectively from FAD in CBO to heme at pH 4.2, but not at pH 5.9.

The results of cytochrome *c* reduction by the pre-reduced CBO suggested that electron transfer from the heme in CBO to cytochrome *c* is also restricted at pH 5.9. The EPR studies of oxidized CBO (data not shown) showed a low-spin state of the heme at both pH 4.2 and 5.9, suggesting that the inactivation of the heme at pH 5.9 might be caused by some conformational changes in the peptide backbone in CBO, rather than by changes of the ligand state of the heme. This inactivation of the heme in CBO is reversible. When the pH is changed from 5.9 to 4.2, the activity of CBO for cytochrome *c* reduction is completely restored.

Several recent reports [5,10,11] suggest that atmospheric oxygen is a poor electron acceptor for CBO, contrary to its nomenclature. The in vivo electron acceptor for CBO still seems to be unknown. Therefore, artificial compounds may, so far, have been utilized as electron acceptors in CBO experiments. The results reported here indicate that the selection of an appropriate electron acceptor is very important, since the pH dependencies of the reduction of FAD and heme are significantly different. It seems inevitable that there is some physiological importance of heme in CBO. The use of an electron acceptor like Cl_2Ind for FAD may lead to erroneous conclusions. Therefore, it is important to use an electron acceptor, like cytochrome *c*, that is specific for the heme component.

The reduction of both FAD and heme in CBO exhibits bi-phasic behavior. This observation indicates that the electron transfer mechanism for cellobiose oxidation by CBO could be rather complicated. This could involve inter-molecular electron transfer between CBO molecules, as is the case in yeast flavocytochrome b_2 [12]. Further experiments are still ongoing to elucidate the electron transfer mechanisms in CBO in more detail.

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