Influence of cellobiose oxidase on peroxidases from *Phanerochaete chrysosporium*

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Reduction of H_2O_2 -oxidized manganese peroxidase (MnP), lignin peroxidase and, to some extent, horseradish peroxidase, was studied in the presence of cellobiose oxidase (CbO) and cellobiose. It was found that the reversion rates for MnP compound II and lignin peroxidase compound II back to native enzymes increased significantly in the presence of CbO and cellobiose. However, the reduction of cytochrome *c* by CbO plus cellobiose was 40 times faster than the reduction of MnP compound II. Also, the lag phase before reversion to the native states decreased for all three

peroxidases in the presence of CbO and cellobiose. Active CbO did not repress formation of compounds I or II of the peroxidases, and Mn^{2+} /veratryl alcohol reduced compound II of the peroxidases much more rapidly than did active CbO. This indicates that, in the presence of Mn^{2+} or veratryl alcohol, MnP and lignin peroxidase can complete their catalytic cycles and function normally without interference from CbO. Without the presence of peroxidase substrates, active CbO reduced compound II of the above peroxidases.

INTRODUCTION

Cellobiose oxidase (CbO) is a sugar oxidase which has been frequently isolated from the white-rot fungus *Phanerochaete chrysosporium* (earlier *Sporotrichum pulverulentum*) [1–6], but is also produced by many other cellulolytic fungi [7], including the brown-rot fungus *Coniophora puteana* [8]. The enzyme oxidizes cellobiose to cellobiono-1,5-lactone during reduction of oxygen, probably to superoxide [2], which in turn can dismutate to H_2O_2 . One or two dioxygen molecules are needed to get one molecule of H_2O_2 . CbO also reduces cytochrome *c*, 2,6-dichlorophenol-indophenol (DCIP) and many quinones. The reduction of cytochrome *c* and quinones is much more rapid than the reduction of oxygen [2,3,9,10].

Cellobiose oxidase is a flavohaemoprotein, whereas the related enzyme cellobiose:quinone oxidoreductase (CBQ) only contains an FAD group. CBQ was isolated in 1974 from both *Trametes (Coriolus) versicolor* and *P. chrysosporium* [11,12]. Cellobiose oxidase and CBQ are produced in greatest amounts in high-nitrogen, cellulose- or wood-meal-containing cultures [1,13,14], which makes the investigation of their possible role in lignin biodegradation difficult. Lignin degradation as well as production of manganese and lignin peroxidases is much better in low-nitrogen glucose cultures [15,16]. The recent finding of lignin biodegradation and production of the above peroxidases together with CBQ in high-nitrogen cellulose media, however, could indicate that both CbO and CBQ are actively influencing lignin biodegradation and peroxidase activity [14]. It is now well documented that both CbO and CBQ can reduce phenoxy radicals and cation radicals produced by manganese and lignin peroxidases [9,10,17,18].

In the present study the influence of pure cellobiose oxidase on the haem group of manganese and lignin peroxidase was investigated. It is reported that reduced CbO converts compound II of these peroxidases into the native state without the presence of Mn^{2+} or veratryl alcohol. The possible importance of this for cellulose and lignin biodegradation is briefly discussed.

MATERIALS AND METHODS

Production and purification of cellobiose oxidase

P. chrysosporium K-3 was grown in a fermenter [19], and CbO was purified as described by Henriksson et al. [4]. Pure CbO was a gift of G. Henriksson, Biomedical Center, Uppsala. Preparation I (S118) contained 2.7 mg in 10 ml of 50 mM ammonium acetate, pH 5, and had an ϵ value of $142 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 420 nm for oxidized CbO ($A_{280} = 0.55$). Preparation II (S178) had an A_{280} of 0.89.

Production and purification of manganese and lignin peroxidase

P. chrysosporium SC26 was cultivated at the Swedish Pulp and Paper Research Institute (STFI) Stockholm, Sweden (with B. Pettersson, STFI) mainly as described [20] and purified by J. Volc, Academy of Sciences of the Czech Republic (AVCR), Prague, Czech Republic. Purity of the peroxidases were tested by isoelectric focusing and SDS/PAGE (Pharmacia Phast Gel). Only one protein band for each enzyme was obtained.

The molarity of the purified manganese peroxidase (MnP) was calculated by using an ϵ_{406} value of $129 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ as noted by Wariishi et al. [21]. The RZ (Reinheit Zahl) purity number value (A_{406}/A_{280}) of our MnP was 4.0. The purified lignin peroxidase (10 mg/l) was calculated to have an ϵ_{408} of $92 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ and an RZ value of 2.8.

Other enzymes

Horseradish peroxidase type VIA was from Sigma, and the stock solution contained 1 mg/ml. It had an ϵ value of $65 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at 404 nm. Catalase from bovine liver (Sigma) contained 11 000 units (one unit is the decomposition of $1.0 \mu\text{mol}$ of H_2O_2 /min at pH 7.0) in 100 mg of solid material. The stock solution contained 1.67 mg/ml. Superoxide dismutase (SOD, Sigma) from bovine erythrocytes contained 4200 units/mg (one unit will inhibit the rate of reduction of cytochrome *c* by 50% in the xanthine + xanthine oxidase system at pH 7.8).

Enzyme assays

Cellobiose oxidase was determined either with 3,5-di-*t*-butyl-*o*-benzoquinone [13] or with DCIP. With DCIP, the assay mixture contained 0.50 (50 μ l) of 0.25 mM cellobiose, 114 μ M DCIP (50 μ l) and 20 mM sodium tartrate, pH 4.5, in a total volume of 2.0 ml. The ϵ_{520} of DCIP in this assay was 7655 M⁻¹·cm⁻¹. The DCIP stock solution contained 4.55 mM DCIP in ethanol/water (2:3, v/v). CbO units are in nkat/litre; 0.25–1.0 mM cellobiose gave the same activity.

Manganese peroxidase was determined either with syringaldazine [13] or with 3-methyl-2-benzothiazoline hydrazone/3-(dimethylamino)benzoic acid as described by Ngo and Lenhoff [22]. The activity of CbO and MnP was further tested by measuring the reduction of the tetramethoxy-azo-*p*-methylenequinone formed from syringaldazine by MnP plus H₂O₂. This quinone was effectively reduced by CbO + cellobiose. CbO without cellobiose did not affect the quinone.

Lignin peroxidase was determined by the method of Faison and Kirk [23], using veratryl alcohol oxidation.

The spectrophotometer used was a Shimadzu UV 160 A set at 23.0 °C. With this standard instrument, the most rapid measurement could be made about 3 s after injection of sample into the cuvette (the sample volume was always 1800–2000 μ l). All experiments were performed in 20 mM sodium tartrate, pH 4.5, which give good MnP activity [24]. The pH of this salt was adjusted with sulphuric acid.

Cytochrome *c* type III (*M_r* 12384) from horse heart was purchased from Sigma. Reduction of cytochrome *c* was measured as the increase in absorbance at 550 nm in 20 mM sodium tartrate, pH 4.5, in which the ϵ value (550 nm) of reduced cytochrome *c* was 26.0 mM⁻¹·cm⁻¹. The reaction mixture contained 1.08 μ M cytochrome *c* (25 μ l of 1 mg/ml) in a total volume of 2.0 ml.

RESULTS

Catalytic cycle of MnP and lignin peroxidase (ligninase)

The reactions for the peroxidases [21,25] are given below:

Both spontaneous and CbO-catalysed reversion of compound II to native peroxidase was studied. Depending on whether AH₂ is a phenolic aromatic substance or a non-phenolic substance like veratryl alcohol, the phenoxy radical AH[•] or the cation radical AH⁺⁺ is obtained. In the case of MnP the reaction (c) is not performed by AH[•] but rather by Mn²⁺ [21].

Influence of CbO on MnP compound II (MnPII)

In order to study the influence of CbO on MnPII the overlay technique in Figure 1 was used. Addition of 3 equiv. of H₂O₂ to native MnP immediately gave MnPII as shown by haem absorbance at 420 nm and 530 nm. MnP compound I or III was not seen. After 9 min, reversion back to native MnP started and was completed after 13–14 min (Figure 1a). In the presence of active CbO (100 μ l, 0.145 μ M) the reversion was much more rapid and started after 60 s as indicated by the decrease in absorbance at 420 nm (Figure 1b). After another 60 s, an increase in absorbance at 407 nm due to formation of native MnP was seen. The reversion was totally completed after 180 s. With 250 μ l of CbO (0.36 μ M) the reversion started after about 80 s (cycle time 20 s) and was completed after 120 s (Fig. 1c).

The MnPII reducing activity of active CbO was measured from the data in Figure 1 as increase in absorbance at 407 nm. The two CbO concentrations used gave 3.6 and 8.1 nkat/litre in MnPII reducing activity. With only H₂O₂ there was a spontaneous rate at 2.2 nkat/litre as measured after start of reversion.

The reducing activity which CbO should catalyse is that of the oxidized MnP, which is 3 μ M H₂O₂/60 s = 50 nkat/litre. If one catalytic cycle of CbO is necessary to produce one molecule of H₂O₂, the two CbO concentrations used (0.145 and 0.30 μ M) will give 26 and 44 μ M H₂O₂ respectively. If the activity against DCIP (1024 nkat/l) is used for calculation, 184 μ M H₂O₂ is produced by the low CbO concentration in 180 s. All these H₂O₂ concentrations want to keep MnPII in the oxidized state and a

Figure 1 Reduction of MnP compound II (420 nm) to native MnP after addition of 3 equiv. of H₂O₂ to 1 μ M MnP (50 μ l) in 20 mM sodium tartrate, pH 4.5, containing 1 mM cellobiose in 2.0 ml total volume

(a) Without CbO; spectra were recorded every minute for 14 min; (b) 100 μ l of CbO (1024 nkat/litre; 0.145 μ M); spectra were recorded every minute for 9 min; (c) 250 μ l of CbO (2560 nkat/litre; 0.36 μ M); spectra were recorded every 20 s for 220 s total time. The overlays were started 4 s after H₂O₂ addition.

Table 1 Reduction rate of cytochrome *c* and peroxide-treated MnP, ligninase and HRP by CbO plus cellobiose in 20 mM sodium tartrate, pH 4.5

	CbO (nkat/l)	Reducing activity (nkat/l)
Cytochrome <i>c</i>	55	14.7
	111	21.4
	222	27.3
	444	76.9
	666	110.8
MnP	0	0.16
	663	3.2
	1024	3.6
	1105	4.7
	2210	10.0
Ligninase	2560	8.1
	3315	11.9
	0	1.25
	552	4.7
HRP	1105	2.9
	0	2.55
	25	5.10

seemingly smaller reducing activity of CbO will be the result, namely the 3.6 and 8.1 nkat/litre given above. If the reduction of oxygen proceeds via the superoxide radical, the above values for H_2O_2 production will be halved.

The absorbance of CbO at the concentrations used did not disturb the haem peak of MnP at 406–420 nm, and the relationship in absorbance between MnP and CbO was 14 and 5.6 for the two concentrations of CbO used. Added Mn^{2+} reduced MnPII immediately and more rapidly than active CbO. Thus Mn^{2+} is the preferred substrate in the catalytic cycle for MnP.

Influence of different amounts of CbO on reduction of MnPII and cytochrome *c*

Time scans measured as disappearance of absorbance at 420 nm or as increase in absorbance at 407 nm were used to calculate the MnPII reducing activity of CbO. With increasing amounts of CbO we found that the start of reduction back to native MnP was quicker and that the reducing activity was greater once started. The results shown in Table 1 with 15, 25, 50 and 75 μl of CbO (663–3315 units) show that MnPII reducing activity was directly correlated with the CbO activity. The lag times before MnPII reduction for the above CbO activities were 130, 100, 30 and 25 s respectively, indicating that CbO positively affected start of reversion to native MnP. Because of some disturbance of the MnPII spectrum with 75 μl of CbO, the MnP concentration was calculated using the ϵ value of $129 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ given for native MnP [21].

In a similar way the reduction of cytochrome *c* by CbO plus cellobiose was calculated. In Table 1 it can be seen that the reduction of cytochrome *c* was much more efficient than the reduction of MnPII, and 40 times more units of CbO was needed to obtain the same rate of MnPII reduction.

Influence of CbO on lignin peroxidase (ligninase)

The influence of CbO on ligninase was investigated both with and without added H_2O_2 . When the native ligninase (407.5 nm)

Figure 2 Oxidation of 20 μl of ligninase by 25 μl of H_2O_2 (2.5 μM , 1.0 equiv.)

(a) Spontaneous reversion to native ligninase; (b) 12.5 μl of CbO (552 nkat/litre; 0.078 μM); (c) 25 μl of CbO (1105 nkat/litre; 0.156 μM). The sodium tartrate buffer contained 0.025 mM cellobiose in a total volume of 1.80 ml. Spectra were recorded once per minute.

was oxidized with 1.0 equiv. of H_2O_2 , an immediate transient formation of compound I was seen. The overlay was started 4 min after H_2O_2 addition. As shown in Figure 2(a), the reversion, probably from a mixture of compounds I and II ($\approx 413 \text{ nm}$), started after (totally) 5 min, took place in small steps and was finished after 15 min. With 12.5 μl and 25 μl of CbO the reversion took place in greater steps and started after 3 and 1 min respectively. The reversion back to 408.5 nm was completed after about 6 min for both CbO concentrations. When measured as increase in absorbance at 408 nm, a decreased reducing activity was obtained with the highest CbO activity (Table 1).

Although active CbO reduced ligninase compound II, this reduction was always much more rapid if veratryl alcohol was added.

Influence of CbO on ligninase without added H_2O_2

During our investigations it was repeatedly found that CbO plus cellobiose bleached the haem of ligninase and changed it to an oxidized state without need for added peroxide (Figure 3a). Little

Figure 3 Oxidation of ligninase by CbO plus cellobiose without the presence of added H_2O_2

(a) 20 μl of ligninase (2.55 μM) was mixed with 150 μl of CbO (6600 nkat/litre; 0.93 μM) and overlay started on addition of 25 μl of cellobiose (0.25 mM); (b) 20 μl of ligninase was added after 15 min incubation of 150 μl CbO with cellobiose. Overlay was started on ligninase addition. Spectra were recorded six times every 30 s.

change in haem wavelength indicated that mainly ligninase compound I was formed. The overlay in Figure 3(a) is very similar to the overlay in Figure 2, indicating that a similar but opposite reaction took place in Figure 3(a).

Since CbO has been reported to produce superoxide radicals and H_2O_2 [2], the above bleaching and compound I formation could be due to oxidation of native ligninase by one or both of these oxygen species. It is possible that native ligninase and superoxide (from CbO + cellobiose) formed a ferric superoxide complex ($\text{Fe}^{3+}\text{--O}_2^{\cdot-}$) absorbing at 412 nm [26]. The presence of 300 units of superoxide dismutase (SOD), however, did not affect the above oxidation of ligninase, indicating that it was not due to superoxide or a superoxide complex.

Figure 3(b) shows that, after incubation of CbO with cellobiose for 15 min to produce superoxide and/or H_2O_2 , the original spectrum of added ligninase ($A = 0.290$ at 408 nm) immediately disappeared and a broad spectrum of oxidized ligninase appeared. The broad peak around 420 nm is composed of both reduced CbO (428 nm) and of oxidized ligninase (412–415 nm). The bleaching (oxidation) was dependent on the incubation time, and less CbO gave less bleaching.

When 100 μl of CbO plus cellobiose was incubated for 15 min in the presence of 4.6 units of catalase, no bleaching or change of added ligninase was obtained. This amount of catalase did not disturb the ligninase spectrum. On the other hand, without catalase, ligninase was again bleached and the wavelength shifted to 414 nm. This latter reaction is comparable with the reaction in Fig. 3(a).

All the above results, including those with SOD and catalase, indicate that native ligninase is oxidized *in vitro* by H_2O_2 produced

Figure 4 Reduction of HRP compound II in the presence or absence of 25 μl of CbO (1105 nkat/litre; 0.156 μM)

(a) The reaction was started by adding 50 μl of H_2O_2 (3.1 equiv.) to 1.62 μM HRP + 0.25 mM cellobiose; (b) the reaction was started by the simultaneous addition of 25 μl of cellobiose and 50 μl of H_2O_2 from two different syringes. Spectrum of native HRP is indicated by an arrow. The overlay scans were run after 0 and 30 s, 1 min and then every minute.

during reduction of CbO by cellobiose. Peroxide production by active CbO has recently been confirmed by Kremer and Wood [27] and by Henriksson (personal communication).

Influence of CbO on horseradish peroxidase (HRP)

When 1.62 μM HRP was incubated with 50 μl of CbO (2210 nkat/litre) plus cellobiose (without peroxide) there was a rapid bleaching of the haem peak at 404 nm and a red-shift to 413 nm after 9 min. This is similar to the results with ligninase (Figure 3a), although the change in wavelength was smaller for ligninase. Since HRP compound II (HRP II) has a Soret band at 420 nm [28], the above experiment probably resulted in a mixture of native HRP and HRP I and II giving the above absorbance at 413 nm due to limited H_2O_2 oxidation. As always in the investigations reported here, the visible region is difficult to use for analytical purposes owing to interference of reduced CbO with the peroxidase peaks.

When HRP was oxidized with 3.1 equiv. of H_2O_2 , HRP II was formed after 30 s, as indicated by a Soret band at 418 nm (Figure 4). According to Marquez et al. [29], as much as 250 equiv. of H_2O_2 is needed to produce HRP compound III, so this species will not be formed here. Both with and without CbO (Figure 4), HRP I was seen directly after H_2O_2 addition. With only H_2O_2 (Figure 4a) the reversion back to native HRP started after 4 min, whereas when H_2O_2 and cellobiose were added simultaneously to HRP + CbO (Figure 4b), the reversion started after 1–2 min. It also took place in greater steps if active CbO was present. The

HRP II reducing activity with or without active CbO was 5.10 nkat and 2.55 nkat/litre respectively, when measured as decrease in absorbance at 420 and 419 nm using the scans in Figure 4. Increased reduction of HRP compound II by active CbO has also been found by G. Henriksson (personal communication).

DISCUSSION

In the present study we have shown that reduced cellobiose oxidase can increase the rate of reversion of the oxidized state compound II of both manganese peroxidase and lignin peroxidase.

In Table 1 the reduction rates of cytochrome *c* and of oxidized Mn-peroxidase, ligninase and HRP are summarized. Several conclusions can be drawn from this Table: (1) Cytochrome *c* is much more easily reduced than the peroxidases; for MnP the difference is 40 times; (2) for MnPII as substrate the rate of reduction increased with increasing CbO activity up to 3000 nkat/litre, while for ligninase compound II there was a tendency for decreased reduction rate with the highest CbO activities; (3) with only H₂O₂ added to obtain oxidized peroxidases, there was a certain spontaneous reversion back to native enzyme (0.16–2.5 nkat/litre); this rate increased significantly in the presence of CbO plus cellobiose, especially for MnP; (4) the start of reduction to native peroxidase was positively influenced by active CbO. The negative influence of strong CbO activity is probably related to H₂O₂ production which decreases the rate of compound II reduction. This was investigated using different amounts of added peroxide (results not shown).

Role of cellobiose oxidase

Our results show that active CbO did not inhibit formation of compounds I or II of MnP or ligninase. Further, Mn²⁺ and veratryl alcohol were preferred substrates for MnP and ligninase respectively, since their effect on reduction of MnPII and ligninase II was much stronger than active CbO. Thus completion of the catalytic cycles for MnP and ligninase can take place without CbO, although CbO may help if Mn²⁺ and/or veratryl alcohol are not present. Whether other substrates like phenols or lignin will reduce compound II and MnP/ligninase better than reduced CbO is not known, although it is highly probable.

We found that the quinone formed from syringaldazine by MnP was effectively reduced by CbO (results not shown) and the radical- and quinone-reducing ability of CbO (and CBQ) is now well-documented [9,10,17,18]. However, Odier et al. [30] reported that their CBQ had no influence on the phenoxyl radical of acetosyringone or on polymerization of guaiacol and synthetic lignin by ligninase. A probable explanation is that Odier added excess CBQ (of which a significant portion could be CbO) which delayed reduction of compound II, giving high ligninase activity and continuous radical production. For the guaiacol and synthetic lignin experiments Odier et al. [30] used as much as 17000 nkat/litre and 170000 nkat/litre of CBQ, which is much more than the 1000 CbO units shown by us to decrease the reduction of oxidized ligninase (Table 1).

There are several possibilities for CbO/CBQ to increase wood degradation in nature. The reduction of radicals and quinones

may decrease polymerization of phenols, lignin and degradation products from lignin. This may increase not only lignin [31], but also cellulose biodegradation [32]. The simultaneous reduction of Fe³⁺ to Fe²⁺ in wood and H₂O₂ production by active CbO [6,27] can give rise to hydroxyl radicals, which are known to degrade at least isolated cellulose [27,33] and possibly also lignin and cellulose in wood [34].

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