# Oxygen reduction by cellobiose oxidoreductase: the role of the haem group

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**Abstract** **We have used optical and electron paramagnetic spectroscopy to study the flavohaem enzyme cellobiose oxidoreductase (CBOR) from *Phanerochaete chrysosporium*. We have examined redox cycles of the enzyme in which the oxidation of cellobiose to cellobionolactone is coupled to the reduction of oxygen. During turnover flavin can reduce oxygen with one electron to produce superoxide or two electrons to produce hydrogen peroxide. Addition of superoxide dismutase significantly extended the time courses of these cycles, slowing the re-oxidation rate of both cofactors. Addition of catalase also affected the haem time course, but to a lesser extent. Experiments in which superoxide was generated in the reaction mixture showed that this radical greatly enhanced the rate of haem re-oxidation. From these results we propose a mechanism in which reactive oxygen species generation by CBOR flavin subsequently re-oxidises CBOR haem. We discuss this mechanism in relationship to the biological function of this enzyme, namely lignocellulose degradation.** **© 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Cellobiose dehydrogenase; Oxidoreductase; Oxygen; Superoxide; Peroxide; Cellulose degradation; Lignin; Reactive oxygen species; *Phanerochaete chrysosporium*

## 1. Introduction

Cellobiose oxidoreductase (cellobiose dehydrogenase, EC 1.1.3.25, CBOR) is a flavohaemoprotein secreted by the white rot fungus *Phanerochaete chrysosporium* and implicated in the degradation of lignocellulose [1].

The flavin domain of CBOR, which is structurally related to glucose oxidase (GOX) [2], catalyses the oxidation of cellobiose to cellobionolactone [3]. The oxidative half of the reaction is analogous to the oxidation of glucose to gluconolactone by GOX, which contains only a flavin cofactor [4,5]. With GOX the two-electron oxidation of glucose is coupled to the two-electron reduction of $O_2$ to $H_2O_2$. The presence of a haem cofactor imparts versatility with regard to electron acceptors; CBOR can additionally reduce one-electron acceptors including cytochrome *c*, semiquinones and ferric iron complexes [6,7] as well as two-electron acceptors such as DCPIP, methylene blue and benzoquinones [1]. There is, however, no generally agreed mechanism for these reactions.

Both aerobic [8] and anaerobic [9] stopped flow experiments have shown that reduction of CBOR flavin and haem cofactors is not synchronous. At pH 6 the flavin cofactor rapidly becomes reduced ($k \leq 20$/s) followed by reduction of the haem cofactor ($k \leq 0.6$/s). The rate of flavin reduction increases with increasing cellobiose concentration [9], consistent with the reaction:

$$\begin{aligned} &\text{cellobiose} + \text{flavin}_{OX}\text{b}_{OX} \rightarrow \\ &\text{cellobionolactone} + \text{flavin}_{RED}\text{b}_{OX} \\ &\text{flavin}_{RED}\text{b}_{OX} \rightarrow \text{flavin}_{SQ}\text{b}_{RED} \end{aligned} \quad (1)$$

The original workers in this field proposed that one-electron reduction of external acceptors involves electron transfer from flavin to haem and subsequent reduction of acceptor by the haem, whereas two-electron reductions involve direct interaction between reduced flavin and acceptor [7,10]. An alternative view [1,11] postulates that both one- and two-electron acceptors react with the flavin. The haem then acts merely as a parking place for extra electrons when one-electron acceptors oxidise the flavin (the 'electron sink' model). In contrast Wilson et al. [12] suggested that even the two-electron acceptor, oxygen, may actually react with the haem group rather than the flavin.

The determination of the X-ray crystal structure for the haem domain of CBOR [13] has confirmed some earlier structural predictions [14] and demonstrated a unique haem-binding β-sheet tertiary structure. But it has not clarified the important problem of haem reactivity.

One of the proposed functions of the enzyme is the degradation of lignocellulose, in cooperation with peroxidases and cellulases [15]. It has been suggested that this is achieved by generating Fenton reagents, especially hydroxyl radicals and related reactive oxygen species (ROS) [16,17]. How oxygen reacts with this enzyme, and the role of the haem group, if any, in this reaction thus has both mechanistic and practical significance. We have therefore reexamined the reactions of CBOR with oxygen.

## 2. Materials and methods

### 2.1. Enzyme preparation

CBOR and the spontaneously occurring flavin domain protein were prepared from the supernatant growth medium of cultures of

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*Abbreviations:* CBOR, cellobiose dehydrogenase (oxidoreductase); SOD, superoxide dismutase; XO, xanthine oxidase; ROS, reactive oxygen species; GOX, glucose oxidase


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*P. chrysosporium* (International Mycology Institute 174727) as described by Jones and Wilson [9]. Under these growth conditions CBOR is the only haem-containing secreted protein. The peroxidases (lignin peroxidase and manganese peroxidase) are produced after longer growth times under slightly different conditions [18]. The $A_{280\,nm}/A_{421\,nm}$ ratio of the enzyme preparation was $<2.0$ which equates to $>85\%$ purity. The enzyme preparation was free from any other chromophores. CBOR concentrations were calculated using $\Delta\varepsilon_{563-571\,nm} = 24.0\ mM^{-1}\ cm^{-1}$, for the dithionite-reduced enzyme. This value is considerably higher than that employed by Jones and Wilson [16] and close to that employed by Kremer and Wood [10].

### 2.2. Spectrophotometry

Steady-state spectrophotometry permitting simultaneous monitoring of flavin and haem reduction was carried out with a Hewlett-Packard diode array spectrophotometer HP8453 linked to a computer. Data analysis was carried out either with the resident HPChem software or by exporting the data as Microsoft Excel files.

### 2.3. Polarography

Oxygen electrode measurements used an Oroboros™ $O_2$ electrode.

### 2.4. EPR system

Electron paramagnetic spectroscopy (EPR) employed a Bruker EMX spectrometer with an Oxford Instruments liquid helium attachment. Data analysis was carried out with the Bruker software package Win-epr™.

### 2.5. Other materials

Catalase was beef liver enzyme from Boehringer. Superoxide dismutase (SOD from bovine erythrocytes) and xanthine oxidase (XO, milk) were from Sigma Chemical Co. Cellobiose and xanthine were Sigma products. Other reagents were of AnalaR™ or similar quality.

## 3. Results

### 3.1. Cellobiose oxidation – EPR studies

We have demonstrated by EPR spectroscopy (at 10 K) the occurrence of the radical species postulated in Eq. 1 and previously found by Cameron and Aust [19]. Under aerobic steady-state conditions the flavin radical with $g = 2.0044$ and a band width of 14.66 gauss is generated, at a maximal concentration of about 20% of total flavin (data not shown). We propose that under aerobic conditions, the $flavin_{SQ}$ formed in Eq. 1 is oxidised either by oxygen or by passing an electron to a re-oxidised *b* haem (see below). Under anaerobic conditions, and with an excess of cellobiose, the enzyme becomes fully reduced, rate-limited by a dismutation reaction between molecules [9].

### 3.2. Cellobiose oxidation kinetics – steady-state analysis

Both intact holoenzyme and isolated flavin domain catalyse oxygen uptake in the presence of cellobiose (data not shown). Catalase markedly reduces the rate of $O_2$ uptake, as previously reported [20]. Fig. 1A shows the spectrophotometric time course of the reduction and re-oxidation of CBOR upon addition of cellobiose, under aerobic conditions. Consistent with earlier work [8,9], the flavin is reduced more rapidly than the haem group. Re-oxidation of the flavin also precedes that of the haem. During the short quasi-steady state the haem is 75% reduced and the flavin is 65% reduced. Under these steady-state conditions the haem is not near redox equilibrium with the flavin. Fig. 1B plots the maximal steady-state reduction of haem as a function of cellobiose concentration together with the corresponding fractional reduction of flavin at the same time point. The steady-state level of reduced haem increases with increasing cellobiose concentration up to a maximum value. Above millimolar cellobiose concentrations the level of reduced haem decreases slightly. Neither prosthetic group becomes fully reduced under aerobic conditions.

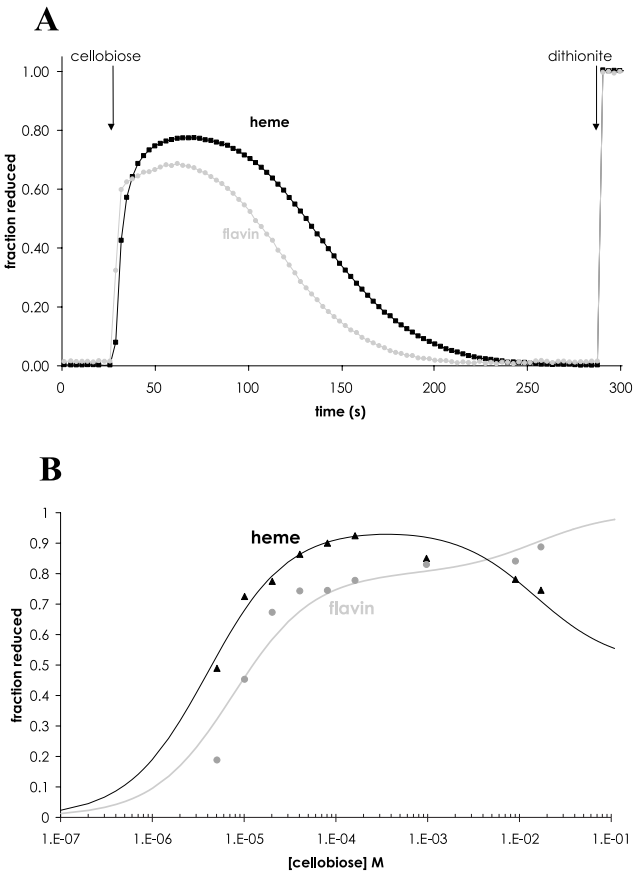



Fig. 1. Oxygen-dependent cellobiose oxidation catalysed by CBOR. A: Time courses of the flavin and haem moieties. Cellobiose (10 μM) was added to CBOR (3.5 μM) in 50 mM air-equilibrated potassium phosphate buffer pH 6.0 at 25°C. The redox states of haem and flavin were monitored at 563–571 nm and 449–571 nm respectively, recording complete spectra every 3 s. Full reduction was achieved by addition of a few grains of sodium dithionite. Time courses are plotted as fractions of flavin and of haem reduced, using the dithionite spectrum as indicating full reduction of both species. B: Haem and flavin reduction vs. cellobiose concentration. Successive additions of cellobiose were added to a sample of CBOR aerobically. Other conditions are as in A.

### 3.3. Cellobiose oxidation: effects of SOD, catalase, superoxide and hydrogen peroxide on the aerobic redox cycles of cellobiose oxidoreductase

Fig. 2 shows the effect of SOD and catalase on the fractional reduction of flavin and haem groups during cycles of reduction and re-oxidation. The duration of each cycle is lengthened by these reagents (cf. Section 4). Fig. 2A shows that the haem *b* re-oxidation rate is slowed when either SOD or catalase is present and markedly slowed when both SOD and catalase are present, although the system remains aerobic. Fig. 2B shows that the flavin redox cycles are lengthened when SOD is present though they are not affected by the addition of catalase. The insets to Fig. 2A,B show the $t_{1/2}$ values under the four conditions employed where $t_{1/2}$ is the time between addition of cellobiose and 50% re-oxidation. SOD increases the fractional reduction of haem and decreases the re-oxidation rate of the flavin. We conclude that both

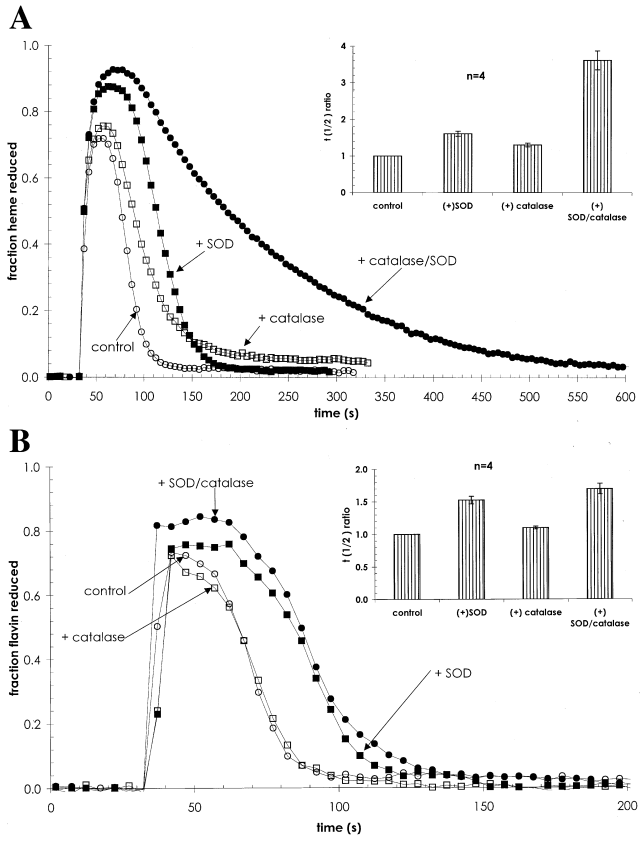



Fig. 2. Oxygen-dependent cycles of cellobiose oxidation by cellobiose oxidoreductase. Effects of SOD and catalase upon the steady state and re-oxidation of the haem (A) and flavin (B). On addition of cellobiose (5 μM, at $t = 30$ s) to CBOR (1.5 μM) in 50 mM potassium phosphate buffer pH 6.0 at 25°C, the enzyme underwent redox cycles as shown in Fig. 1A. Other conditions are as follows: (○) control – no further additions; (■) plus superoxide dismutase (50 U/ml bovine erythrocyte enzyme); (□) plus catalase (30 nM bovine liver enzyme); and, (●) plus both SOD and catalase. Results are plotted as fraction haem reduced VS time. Each cycle was repeated three times. Full reduction was obtained by dithionite addition. Spectra were recorded every 5 s. The redox state of the haem group was monitored at 563–571 nm. The inset shows the time ($t_{1/2}$) for each of these redox cycles to become 50% re-oxidised, normalised to $t_{1/2}$ for the control. With the exception of the flavin redox cycle with catalase all are significantly different from the control ($P < 0.01$).

hydrogen peroxide and superoxide are being generated during the catalytic cycle and that either can re-oxidise the haem. Fig. 3 confirms these interpretations. In Fig. 3A the addition of xanthine and XO (a convenient source of superoxide), in the presence of catalase, is used to modify the haem and flavin cycle times and haem re-oxidation rates. The figure shows the haem and flavin cycles, together with the rate of superoxide production. Superoxide formation was monitored by the reduction of cytochrome *c* in a parallel experiment with the same concentrations of xanthine and XO. Superoxide evidently causes a marked increase in haem re-oxidation rate, presumably according to:

$$\text{CBOR haem } b^{2+} + O_2^{\bullet -} + 2H^+ \rightarrow \text{CBOR haem } b^{3+} + H_2O_2 \quad (2)$$

Fig. 3B shows the effect of hydrogen peroxide on the re-oxidation of haem. Catalase and SOD were included in this experiment in order to lengthen the re-oxidation time course of the haem (as in Fig. 2A). After the flavin had been re-oxidised the haem was still ~90% reduced. This allowed observation of haem oxidation by hydrogen peroxide. After inhibition of catalase by azide, any remaining peroxide was free to re-oxidise the haem, and the oxidation rate doubled. Addition of excess $H_2O_2$ to partially reduced haem caused complete re-oxidation. Such haem oxidation may lead to the formation of hydroxyl radicals, as in:

$$\text{CBOR haem } b^{2+} + H_2O_2 + H^+ \rightarrow \text{CBOR haem } b^{3+} + H_2O + OH^{\bullet} \quad (3)$$

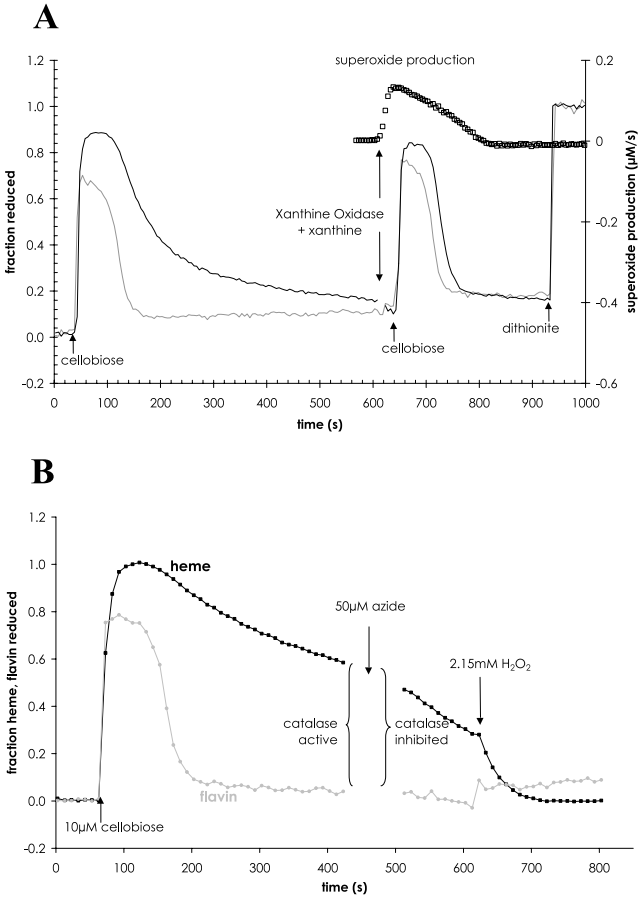


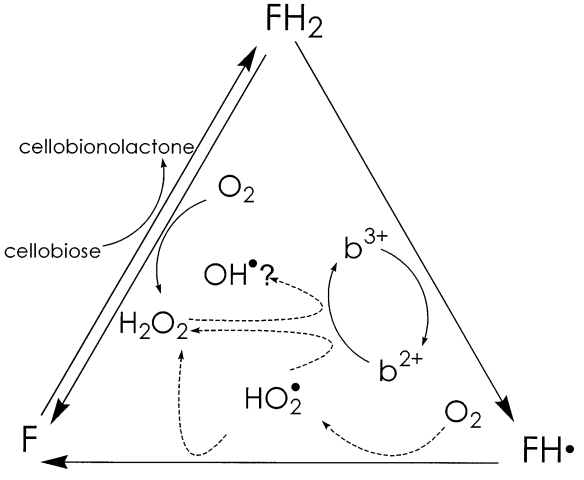

Fig. 3. Effects of ROS on cellobiose oxidoreductase redox cycles. A: Effects of xanthine and XO upon the steady state and re-oxidation of the haem group. Redox cycles of CBOR (1.25 μM), in 50 mM air-equilibrated potassium phosphate buffer/100 μM DTPA pH 6 at 25°C, were initiated by addition of cellobiose (20 μM aliquots) in the presence of catalase (75 nM). Superoxide was generated in situ by the addition of xanthine (50 μM)/XO (20 nM) at $t = 620$ s. Other conditions are as in Fig. 1A. (Grey line) fractional flavin reduction (449–571 nm); (black line) fractional haem reduction (563–571 nm); (□) production of superoxide (monitored by reduction of cytochrome *c* in a parallel experiment). B: Effects of hydrogen peroxide upon the steady state and re-oxidation of the haem group. CBOR (1 μM) was reduced with 10 μM cellobiose in the presence of SOD (50 U/ml) and catalase (75 nM). After re-oxidation of the flavin, azide (50 μM) was added (450 s) to inhibit catalase. $H_2O_2$ (2.15 mM) was added at 630s. 50 mM potassium phosphate buffer pH 6.0/100 μM DTPA at 25°C. (Black line) Fractional haem reduction (563–571 nm); (grey line) Fractional flavin reduction (449–571 nm).

## 4. Discussion

The CBOR aerobic steady state in which the haem is only partially reduced led Wilson et al. [12] to suggest that the haem could react directly with molecular oxygen. However, the redox potential of CBOR haem (+165 mV) [10] makes one-electron reduction of oxygen highly unfavourable. As the Eo′ for the $O_2/O_2^{\bullet-}$ couple is −170 mV in molar terms, the equilibrium constant for such a one-electron reduction would be almost $10^{-6}$. There are no extra redox centres available for two- or four-electron oxidoreductions typical of classical oxidases, and hexacoordination of the haem iron [13,14] precludes oxidation via a haem ferryl state.

We have interpreted our results in Scheme 1 which presents an overall mechanism for oxygen reduction by the enzyme. The lengthened haem and flavin redox cycles in the presence of SOD (Fig. 3) are explained by a one-electron oxidation of flavin semiquinone radical by molecular oxygen. Thus when superoxide is not available to re-oxidise the haem, the latter cannot accept electrons from the flavin and the cycle is slowed (Fig. 2). This scheme provides a solution to the problem of how oxygen reduction can occur with partial oxidation of the haem without a requirement for direct reaction between haem and molecular oxygen, namely that only the flavin domain is required to react directly with oxygen. One product is hydrogen peroxide; another is superoxide. Re-oxidation of the haem group is achieved by either. Both catalase and SOD thus slow haem re-oxidation.

Klinman [21] has analysed a number of oxygen activation processes in terms of an initial formation and stabilisation of a superoxide anion. The unique character of CBOR thus lies not in the generation of peroxide and superoxide by oxygen reduction, but in their subsequent reactivities with the second redox centre in the enzyme, the haem.




Scheme 1. A mechanism for generation of ROS by CBOR. Oxidised flavin (F) is reduced to $FH_2$ by the $2e^-$ oxidation of cellobiose to cellobionolactone. Oxidation of $FH_2$ can occur in either of two ways: (i) a single $2e^-$ oxidation by $O_2$ yielding $H_2O_2$ or (ii) two successive $1e^-$ oxidations by haem $b^{3+}$ and by $O_2$ yielding haem $b^{2+}$ and superoxide $O_2^{\bullet-}$, respectively. The first $1e^-$ step produces flavin semiquinone (FH•) as an intermediate and the second $1e^-$ step returns flavin to its fully oxidised state. ROS generated from reduction of oxygen by $FH_2$ or by FH• oxidise haem $b^{2+}$ to $b^{3+}$. The redox cycle is slowed when ROS are removed. Dashed lines represent alternative $1e^-$ oxidation steps.

We conclude that the flavin may produce hydrogen peroxide and superoxide by reduction of oxygen. When reduced haem reacts with ROS to regenerate oxidised haem it produces $H_2O_2$ (if challenged with $HO_2^{\bullet}$) or possibly $HO^{\bullet}$ (if challenged with $H_2O_2$). Although the enzyme may be capable of producing the end product of Fenton chemistry, i.e. $HO^{\bullet}$, without participation of ferrous iron species, it is unlikely that $HO^{\bullet}$ could migrate away from the active site, given its reactivity. CBOR haem is known to reduce ferric iron species, such as ferric oxalate [6], that can then react with peroxides, generating hydroxyl radicals or ferryl iron, both powerful oxidants. At present we favour the view that this single enzyme can produce both reagents required for Fenton chemistry, i.e. $H_2O_2$ and mobile ferrous iron complexes, that may migrate into and disrupt the lignocellulose matrix. Further experiments will be needed to confirm the extent of these reactions under the conditions in which the enzyme normally functions.

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