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Conversion of O_2 species by cellobiose dehydrogenase (cellobiose oxidase) and glucose oxidase – a comparison

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Cellobiose dehydrogenase from Phanerochaete chrysosporium produces H_2O_2 by electron transfer between cellobiose and O_2 with a lower yield than the 1:1:1 molar ratio displayed by Aspergillus niger. glucose oxidase in the similar reaction between glucose and O_2. The discrepancy could best be explained if both a Fenton's reaction and the spontaneous reactivity of the oxygen species formed were taken into account.

Introduction

White rot fungi such as *Phanerochaete chrysosporium* produce, in addition to cellulases, many other extracellular enzymes under cellulolytic conditions. One of these is cellobiose dehydrogenase (CDH), earlier named cellobiose oxidase. CDH carries one heme of the cytochrome *b* type and one FAD (Morpeth, 1985), and absorbs specifically to cellulose by a binding site separate from the catalytic site (Renganathan *et al.*, 1990; Henriksson *et al.*, 1991). CDH has recently been shown to have a cigar-shaped structure (Lehrner *et al.*, 1996).

Beside cellobiose, lactose and water-soluble cellodextrins are good electron donors for cellobiose dehydrogenase with lactones as products (Ayers *et al.*, 1978). Several electron acceptors have been reported: complexed ferric ions (Kremer and Wood, 1992a), quinones (Westermark and Eriksson, 1974), O_2 (Morpeth, 1985) and phenoxy cation radicals (Ander *et al.*, 1990). The biological function of CDH is unknown, but the following three options have been discussed:

1) Supporting Fenton's reaction (hydrogen peroxide and Fe^{2+} (Fenton, 1894) that produces reactive agents taking part in wood degradation (Kremer and Wood, 1992b; Henriksson *et al.*, 1995). The precise nature of these agents is controversial, but a hydroxyl radical, a peroxo complex of Fe(II) and FeO^{2+} have been suggested as candidates (Wink *et al.*, 1994). Under aerobic conditions, an active Fenton's reaction requires a steady production of Fe^{2+}, which can be carried out by CDH in the presence of cellobiose as electron donor and hydrogen peroxide that can be produced by CDH itself, or by *P. chrysosporium* oxidases.

2) The enzyme inhibits repolymerization of aromatic radicals created by the action of lignin peroxidases. As a result, the accessibility of the cellulose may be enhanced (Ander *et al.*, 1990; Henriksson *et al.*, 1993). It is also possible that aromatic radicals can be produced by Fenton's reaction in wood.

3) Co-operation with manganese peroxidase to make Mn(II) available by reducing $Mn(IV)O_2$ and, at the same time, forming suitable complexing agents for the reactive Mn(III) ion (Roy *et al.*, 1994).

Both hydrogen peroxide and superoxide anion have been suggested as primary reduction products of molecular oxygen (Morpeth, 1985; Kremer and Wood, 1992a). These oxygen species have been suggested to take part in wood degradation by both brown and white rot fungi (Faison and Kirk, 1983). The fact that CDH readily reduces oxidized 2,2-azino-bis(3-ethylbenzthiazolin-6-sulphonic acid) (Henriksson *et al.*, 1993) as well as compound 2 of horseradish peroxidase has been a source of confusion (Ayers *et al.*, 1978; Odier *et al.*, 1988), since the horseradish peroxidase/2,2-azino-bis (3-ethylbenzthiazolin-6-sulphonic acid) system is a popular analysis method for hydrogen peroxide. It is clear, however, that CDH produces hydrogen peroxide in the presence of an electron donor and molecular oxygen and can also degrade hydrogen peroxide under the same conditions (Henriksson *et al.*, 1993). In this work we compare the stoichiometry of CDH with

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the well characterised hydrogen peroxide-producing enzyme, glucose oxidase (Wilson and Turner, 1992), in order to investigate the potential pathways and significance of the dioxygen reduction of CDH.

Materials and methods

Materials

Cellobiose, o-metoxyphenol (guaiacol) and desferrioxamine were from Sigma, USA. The GP343-oxygen electrode was from EDT instruments, UK.

Enzymes

Cellobiose dehydrogenase with A420/A280 > 0.62 was purified from cellulolytic cultures of *Phanerochaete chrysosporium* strain K3 as described in (Henriksson *et al.*, 1991). Glucose oxidase from *Aspergillus niger*, superoxide dismutase, horseradish peroxidase and catalase were from Sigma, USA.

Methods of assay

O_2 consumption was measured with a GP343 O_2-electrode. The concentration of H_2O_2 was determined by mixing 0.9 ml of sample with 4.2 ml of 0.02 μM horseradish peroxidase using 2 mM guaiacol as a substrate in 50 mM sodium acetate, pH 5.0, and monitoring at 440 nm. Reducing ends of cellobiose were assayed using the Somogyi-Nelson method (Somogyi, 1952). H_2O_2 was previously decomposed by catalase to avoid possible disturbance of this method. Calculations were performed using the program SIMFIT.

Experimental conditions

CDH (0.067 μM) and cellobiose (0.25 mM) were mixed with the appropriate chemicals at 30°C in 50 mM sodium-acetate, pH 5. The oxygen concentration was kept stable at 235 μM (saturation at 30°C) by continuous aeration. The aeration was disrupted for short intervals to allow monitoring of the O_2 consumption rate. Reaction was observed by simultaneous measurement of H_2O_2, cellobiose and O_2 concentration. Aliquots for determination of H_2O_2 and cellobiose were withdrawn and boiled for two minutes to stop the reaction. This is necessary, since active CDH interferes with peroxidases and possibly also with the dye (Henriksson *et al.*, 1993) and boiling does not influence the hydrogen peroxide level to a measurable extent. Similar experiments were performed with glucose oxidase and glucose.

Results

CDH formed hydrogen peroxide in the presence of cellobiose and molecular oxygen. Cellobiose and oxygen were consumed in practically equimolar amounts, but the quantity of hydrogen peroxide detected in the reaction mixture was significantly lower and seemed to reach pseudo-steady-state conditions (Fig. 1a). In a similar experiment with glucose oxidase and glucose the stoichiometry between glucose and dioxygen consumed and hydrogen peroxide produced (Fig. 1b), was close to 1:1:1 (0.9:0.9:1.1).

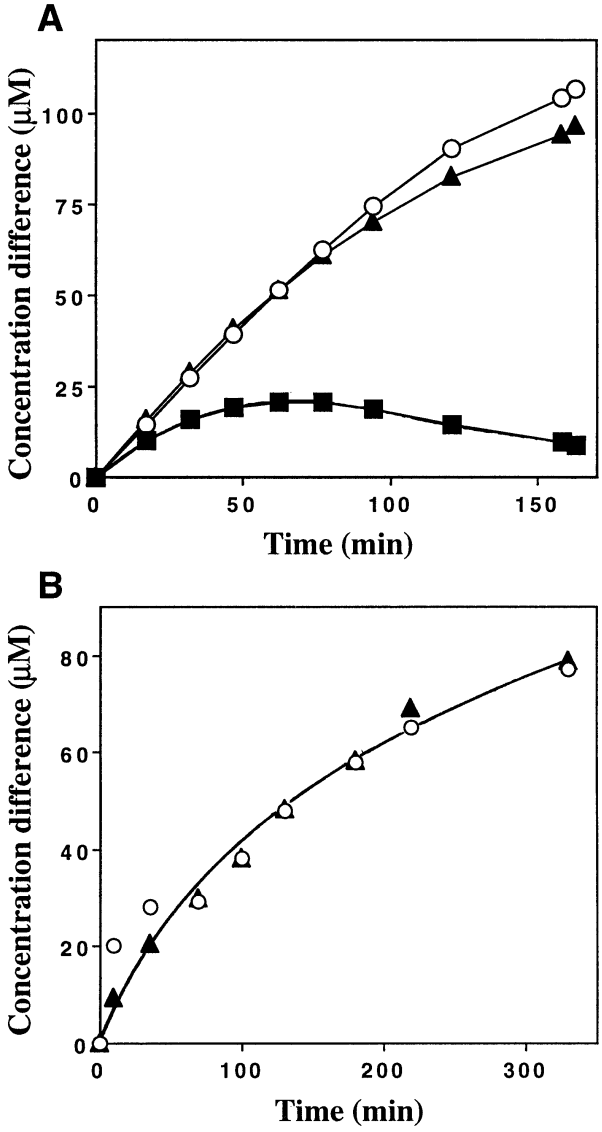

Figure 1 Balance of saccharide oxidation by glucose oxidase and cellobiose dehydrogenase. A) Cellobiose dehydrogenase: Hydrogen peroxide formed (■). consumed cellobiose (○) and oxygen (▲). The reaction was performed as described in the text. B) Glucose oxidase: Consumed oxygen (▲), consumed glucose (○) and formed hydrogen peroxide (■). The initial mixture included 1 mM glucose, 0.25 U/ml glucose oxidase.

In the presence of catalase, the rates of consumption of cellobiose and oxygen by CDH were constant during the experiment (Fig. 2) with a ratio cellobiose/oxygen consumed of 2.1 ± 0.1. No hydrogen peroxide was detected.

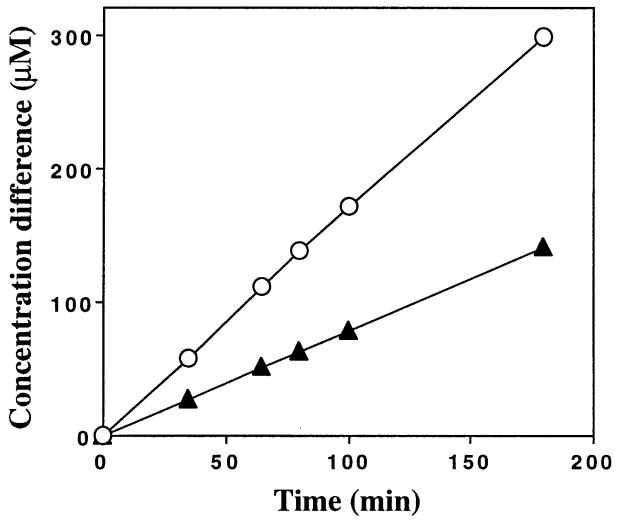

Figure 2 The balance of cellobiose oxidation in the presence of catalase. (○) consumed cellobiose, (▲) consumed oxygen. The concentration of catalase was 1.6 mg/ml.

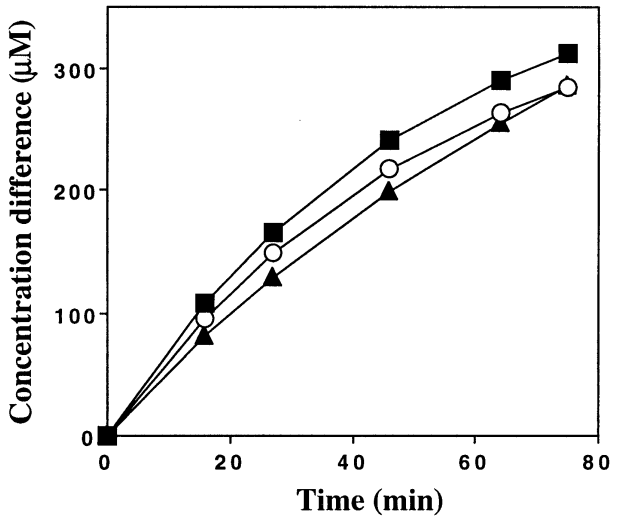

Figure 3 Formation of hydrogen peroxide and degradation of cellobiose by CDH in the presence of 1 mM desferrioxamine. (▲) consumed cellobiose, (○) formed hydrogen peroxide. The concentrations of other reagents were the same as described in the text.

Since the solutions inevitably contain traces of ferric ions, we repeated the experiments using 1 mM desferrioxamine mesylate to inactivate any traces of ferric ions participating in hydrogen peroxide degradation (Kremer and Wood, 1992a). Under these conditions hydrogen peroxide is formed stoichiometrically from cellobiose. The stable ratio between cellobiose oxidised and hydrogen peroxide formed was 1.0 ± 0.1 (Fig 3).

Discussion

Glucose oxidase formed hydrogen peroxide from O_2 during glucose oxidation in an expected 1:1:1 stoichiometry (Fig 1b). The close fit to this ideal ratio demonstrates the accuracy of the method used. The reaction proceeds according to the following scheme.

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconolactone} + H_2O_2 \quad (1)$$

The case of CDH is different. In analogy with glucose oxidase it takes up two electrons from the electron donor to form a lactone (Ayers *et al.*, 1978), but hydrogen peroxide is not produced in equimolar amounts (Fig 1a), i.e., it does not accumulate. There are several reactions that can be responsible for the disappearance of hydrogen peroxide.

a) Traces of metal ions can decompose hydrogen peroxide.

$$H_2O_2 \xrightarrow{Me^{+/2+}} H_2O + O_2 \quad (2)$$

During pseudo-steady state conditions, i.e. hydrogen peroxide concentration is constant, the consumption rates of cellobiose and dioxygen should have a ratio of 2:1.

b) In principle a peroxidase reaction could also take place:

$$2H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O_2 \quad (3)$$

The ratio between the consumption rates of cellobiose and dioxygen should also here be 2:1

c) Hydrogen peroxide can be degraded according to Fenton's reaction.

$$2H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^- + Fe^{3+} \quad (4)$$

The responsibility of hydroxyl radicals for the reactivity of Fenton's reagent has been questioned (Wink *et al.*, 1994). However, also alternative reactive agents are expected to provide similar overall results. The Fe^{2+} can be created by CDH according to:

$$\text{Cellobiose} + 2Fe^{3+} \xrightarrow{\text{CDH}} \text{Cellobionolactone} + 2Fe^{2+} \quad (5)$$

Here we must bear in mind that iron is normally Fe^{3+} in an aerobic environment. Therefore, the ferrous ions necessary for a Fenton's reaction do, in such a case, represent a non-equilibrium condition that is subject to kinetic rather than thermodynamic control. The steady flow of electrons from cellobiose forms the basis for the non-equilibrium state. Furthermore, the reaction rate for reduction of Fe^{3+} ions is high enough to create a 'useful' concentration of ferrous ions even in the presence of a great excess of molecular oxygen as a competing electron acceptor. Similarly, hydrogen peroxide competes favourably with dioxygen to accept electrons from ferrous ions. If we assume that the hydroxyl radicals combine quantitatively to H_2O_2, the consumption rates of cellobiose and dioxygen should also in this case have a ratio of 2:1. However, one has to take into account the high reactivity of the obtained hydroxyl radical. If we assume that all radicals 'disappear', the ratio, consumed cellobiose: dioxygen consumption rate should be 3:2. This is closer to the 1:1 obtained (Fig. 1a) than are the other models, but still not satisfactory. A likely target for the hydroxyl radicals is cellobiose, the most abundant organic molecule in the assay system (Adams and Wilson, 1969; von Sonntag, 1980). Kremer and Wood (1992b) demonstrated that an ongoing Fenton's reaction leads to oxygen uptake in the presence of cellulose. They proposed the following reaction scheme, where HROH is a part of a saccharide:

$$OH^{\bullet} + HROH \rightarrow H_2O + {}^{\bullet}ROH \quad (6)$$

$$O_2 + {}^{\bullet}ROH \rightarrow R{=}O + HO^{\bullet}{}_2 \quad (7)$$

This reaction scheme may explain the high consumption of O_2 by CDH, and thus the most probable pathway for the reaction of this system is the formation of hydrogen peroxide in parallel to (1) followed by [(4) to (7)]. This conclusion is further supported by the experiment in the presence of the chelator desferrioxamine, which will inactivate ferric ions. Here, hydrogen peroxide was produced from cellobiose and oxygen by CDH with 1:1 stoichiometry (Fig 3). Therefore, in the absence of reactive ferric ions, hydrogen peroxide is the only product of oxygen reduction by CDH and no further reactions seem to take place. This experiment also demonstrates that CDH readily produces hydrogen peroxide without the suggested involvement of Fe^{2+} (Wood, 1993). It is important to note that it is not the complexation of ferric ions that inhibits the hydrogen peroxide degradation, but rather that the special desferrioxamine complex cannot participate, whereas, e.g., cyanide complexes readily do (Henriksson *et al.*, 1993). Furthermore, no simple ferric ions $[Fe(H_2O)_6^{3+}]$ are available under these reaction conditions. A similar conclusion can be drawn from the experiments including catalase (Fig. 2). Here, further reduction of hydrogen peroxide is suppressed and all hydrogen peroxide decomposes without net uptake of electrons from cellobiose because of the fast degradation of hydrogen peroxide by catalase:

$$2H_2O_2 \rightarrow H_2O + O_2 \quad (8)$$

We found that the amount of oxygen consumed was half that of cellobiose, which further verifies that the oxygen consumed is mainly converted into hydrogen peroxide. Indeed, CDH can donate electrons to both Fe^{3+} and O_2 at the same time. This leads to the enhanced Fenton's reaction whereby the enzyme creates both components of Fenton's reagent. Obviously, even traces of Fe^{3+} are enough to catalyse hydrogen peroxide degradation, which explains the results published by Henriksson *et al.* (1993).

Biological/technical significance

It is known that oxygen radicals often occur accidentally in biological systems, but the distinct difference between the behaviour of CDH and glucose oxidase in this aspect indicates that CDH indeed has a design favourable for production of reactive oxygen species. This work explains the hydrogen peroxide degradation reported by Henriksson *et al.* (1993), and shows that CDH is capable of directly reducing dioxygen independently of iron ions. In an earlier work it was shown that CDH in the presence of hydrogen peroxide and various Fe(III) complexes could degrade cellulose, xylan and lignin (Henriksson *et al.*, 1995). The concentrations of Fe^{3+} and H_2O_2 were, however, higher than probably could be expected *in vivo*. The present study illustrates that CDH is capable of generating the hydrogen peroxide needed and can form reactive agents even if only traces of Fe(III) are present. The reaction between dioxygen and radicals induced on the carbohydrates induce superoxide anions (7) that can cause further degradation in chain reactions. Degradation of xenobiotics has been suggested as an application of white rot fungus. The interest has so far mainly been focused on manganese and lignin peroxidases, but the generation of Fenton's reagent by CDH may also play a role for broad spectra degradation.

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