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Mechanisms of redox interactions between lignin peroxidase and cellobiose:quinone oxidoreductase

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The mechanism of redox interactions between the heme-enzyme, lignin peroxidase (LiP), and the FAD-enzyme, cellobiose:quinone oxidoreductase (CBQ) (EC 1.1.5.1), was investigated under various conditions. Veratryl alcohol oxidation by LiP was inhibited by CBQ in the presence of cellobiose. Lineweaver-Burk plots at various CBQ concentrations suggest that this inhibition is non-competitive. The oxidation rate of the reduced CBQ ($FADH_2$) by LiP plus H_2O_2 increased significantly only in the presence of veratryl alcohol. Furthermore, the cation radical derived from 1,2,4,5-tetramethoxybenzene was reduced by CBQ in the presence of cellobiose. It is concluded from these results that CBQ can reduce aromatic cation radicals and that veratryl alcohol acts as a radical mediator of the redox interactions between LiP and CBQ.

Lignin peroxidase; Cellobiose:quinone oxidoreductase; Flavoprotein; *Phanerochaete chrysosporium*

1. INTRODUCTION

Cellobiose:quinone oxidoreductase (CBQ) (EC 1.1.5.1) was discovered and isolated from a white-rot fungus *Sporotrichum pulverulentum* (*Phanerochaete chrysosporium*) by Westermark and Eriksson [1,2]. CBQ catalyses the oxidation of cellobiose to cellobiono-δ-lactone in the presence of a quinone as an electron acceptor. The prosthetic group of CBQ, FAD, plays the role of electron mediator between cellobiose and the quinone. CBQ is induced by degradation products of cellulose [1], and has a cellulose binding-domain similar to those of endo-glucanases [3,4]. It is, therefore, reasonable to assume that CBQ plays some important role in cellulose biodegradation. Although the physiological function of the quinone-reducing activity by CBQ is still ambiguous, CBQ might also have some role in lignin biodegradation.

The redox interactions between CBQ, laccase, and peroxidases have already been investigated. Odier et al. [5] reported that CBQ has no effect on phenoxy-radical coupling reactions catalyzed by lignin peroxidase (LiP). However, Ander et al. [6] demonstrated that CBQ significantly inhibits LiP catalyzed polymerization of Kraft lignin and decarboxylation of vanillic acid by laccase and several peroxidases, as well as oxidation of veratryl alcohol and 1,2,4,5-tetramethoxybenzene by LiP. Furthermore, Bao et al. [7] recently reported that CBQ inhibits the peroxidase-catalyzed oxidation of iodide to triiodide and suggested that this inhibition is due to a two-electron reduction of triiodide by CBQ in the presence of cellobiose.

In the present work, the mechanism of redox interactions between LiP and CBQ was investigated. We have demonstrated the cation radical reducing-activity of CBQ, as well as the role of veratryl alcohol as a radical mediator between the LiP and CBQ redox cycles.

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2. MATERIALS AND METHODS

2.1. *Preparation of cellobiose:quinone oxidoreductase (CBQ)*

Phanerochaete chrysosporium strain K-3 was grown in a fermentor on modified Norkrans medium [8], containing 2% crystalline cellulose (w/v) as a carbon source, for 4 days at 37°C.

CBQ was purified according to Westermark and Eriksson [2]. Additionally, Mono-Q FPLC column chromatography (Pharmacia-LKB) was used for further purification. CBQ was eluted with a linear gradient of 0–500 mM NaCl in 80 ml of 10 mM Tris-HCl (pH 8.5). The purified CBQ conformed a single band of mol. wt. 75 000 based on SDS-PAGE analysis. The isoelectric point was determined to be 6.4, although a minor band at 5.8 was apparent (data not shown). The visible spectrum of the purified CBQ was typical for a FAD enzyme, and showed no heme absorption. All electrophoresis was performed on a PHAST system (Pharmacia-LKB) in the appropriate media according to the manufacturer's instructions. The specific activity of the purified CBQ with dichlorophenol–indophenol (DCPIP) as substrate was 28.5 μkat/μmol at pH 4.5.

2.2. *Preparation of lignin peroxidase (LiP)*

A partially purified LiP was kindly provided by Dr. R.L. Farrell (Repligen Sandoz-Research Corporation, Lexington, MA). Further purification was carried out using Mono-Q FPLC column chromatography [9]. The major peak corresponding to LiP H-2 was collected. The purified LiP H-2 migrated as a single band upon SDS-PAGE with an apparent mol. wt. of 38 000 (data not shown). The specific activity of the purified LiP with veratryl alcohol as substrate was 8.0 μkat/μmol at pH 4.5.

2.3. *Enzyme assays*

All enzyme assays were carried out in 50 mM sodium tartrate buffer

(pH 4.5) at 30°C. CBQ activity was measured in a solution of 500 μM cellobiose and 75 μM dichlorophenol-indophenol as the decrease in absorbance at 600 nm (ε = 2.5 $mM^{-1}\cdot cm^{-1}$). LiP activity was measured in a solution containing 200 μM H_2O_2 and 500 μM veratryl alcohol as the absorbance increase at 310 nm due to veratraldehyde production (ε = 9.3 $mM^{-1}\cdot cm^{-1}$).

2.4. *Inhibition of LiP activity by CBQ*

Inhibition of LiP activity was measured in 1000 μl of 50 mM sodium tartrate buffer (pH 4.5) at various concentrations of veratryl alcohol plus 500 μM cellobiose in the presence of different amounts of CBQ (0, 0.022, 0.044 and 0.089 μM). The same amount of LiP (0.42 nkat) was used in each measurement. Kinetic constants, K_m for veratryl alcohol and K_i for CBQ, were estimated from Lineweaver-Burk ($1/v$–$1/[S]$) and K_m/V_{max}– [I] plots, respectively.

2.5. *Oxidation of reduced CBQ by LiP*

The reduced form of CBQ ($FADH_2$) was prepared by addition of 5 μl of 2 mM cellobiose to CBQ (1.2 nmol) in 795 μl of 50 mM sodium tartrate buffer (pH 4.5) at 30°C. After 3 min of incubation, LiP (0.67 nkat) in 200 μl of the same buffer plus H_2O_2 (0.2 μmol), without or with veratryl alcohol (0.5 μmol), was added. The oxidation of $FADH_2$ in CBQ to FAD was measured as increased absorbance at 457 nm.

2.6. *Reduction of the 1,2,4,5-tetramethoxybenzene cation radical by CBQ*

1,2,4,5-Tetramethoxybenzene was kindly provided by Dr. T.K. Kirk (Forest Products Laboratory, USDA, Madison, WI). 1,2,4,5-Tetramethoxybenzene (100 nmol), in 990 μl of 50 mM sodium tartrate buffer (pH 4.5) containing cellobiose (500 nmol), was converted to the cation radical using 67 nkat of horseradish peroxidase (Sigma) and H_2O_2 (80 nmol) at 30°C. After 15 s, 10 μl of the same buffer, with or without CBQ (2.1 nkat), was added. The change in absorbance at 450 nm due to the reduction of the cation radical was measured.

3. RESULTS

3.1. *Inhibition of LiP activity (veratryl alcohol oxidation) by CBQ*

In the presence of cellobiose, CBQ significantly inhibits veratryl alcohol oxidation by LiP. The inhibitory effect of CBQ is dependent upon the amount of CBQ added. Lineweaver-Burk plots of LiP activity in the presence of increasing amounts of CBQ plus cellobiose indicated that the inhibition is typically non-competitive (Fig. 1) since the K_m for veratryl alcohol is constant (170 μM) with different amounts of CBQ. Furthermore, K_i for CBQ was estimated to be 0.08 μM.

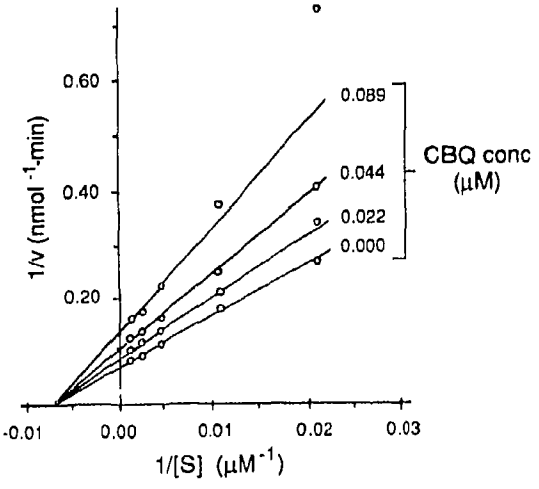

Fig. 1. Lineweaver-Burk plots for veratryl alcohol oxidation by LiP in the presence of increasing amounts of CBQ plus cellobiose (500 μM). $1/v$, reciprocals of veratraldehyde formed ($nmol^{-1}\cdot min$). $1/[S]$ reciprocals of veratryl alcohol concentration (μM^{-1}).

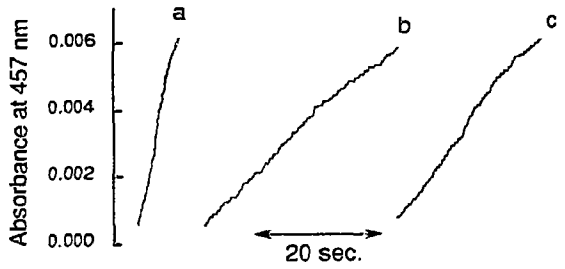

Fig. 2. Oxidation of $FADH_2$ in CBQ by the LiP system. (a) LiP + H_2O_2 + veratryl alcohol. (b) LiP + H_2O_2. (c) Control (without H_2O_2).

3.2. *Oxidation of the reduced CBQ by LiP*

The prosthetic group in CBQ (FAD) typically has an absorption peak at 457 nm, which disappears when it is reduced to $FADH_2$. Therefore, the redox state of CBQ can be estimated by measuring the absorption changes at 457 nm. Reduced CBQ ($FADH_2$) was prepared by addition of a limited amount of cellobiose, and oxidation of $FADH_2$ to FAD by LiP plus H_2O_2 was studied in the presence and absence of veratryl alcohol (Fig. 2). The $FADH_2$ in CBQ was oxidized to FAD under atmospheric conditions without addition of LiP at a rate of 2.3 nmol/min. When LiP plus H_2O_2 was added to the CBQ solution, a slight decrease of the oxidation rate (1.9 nmol/min) was observed. However, when veratryl alcohol was added to the system, the oxidation rate of $FADH_2$ increased significantly (7.5 nmol/min).

3.3. *Reduction of the 1,2,4,5-tetramethoxybenzene cation radical by CBQ*

1,2,4,5-Tetramethoxybenzene is oxidized very rapidly to its cation radical by peroxidases. The cation radical

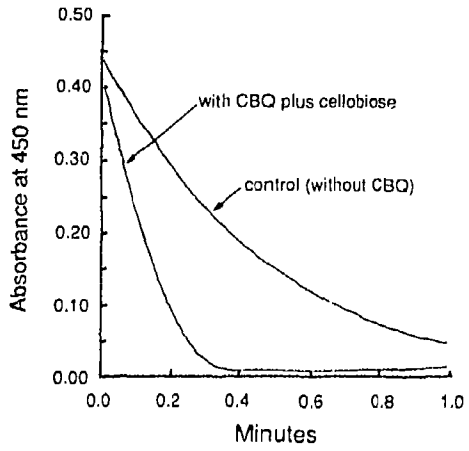

Fig. 3. Reduction of 1,2,4,5-tetramethoxybenzene cation radical by CBQ plus cellobiose.

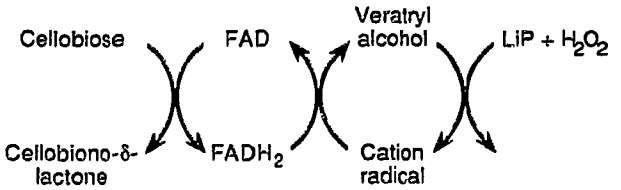

Fig. 4. Mechanisms of redox interactions between LiP and CBQ in the oxidoreductation of veratryl alcohol.

formed has a yellow color. Although this cation radical decays gradually, its stability is still enough to allow investigation of the radical scavenging effect of CBQ by monitoring the decreasing absorbancy at 450 nm. As shown in Fig. 3, in the presence of cellobiose, CBQ speeds reduction of the cation radical.

4. DISCUSSION

Inhibition of LiP activity by CBQ is dependent upon the presence of cellobiose [6], suggesting that the reduced form of CBQ ($FADH_2$) is required for inhibition. Therefore, the reducing ability of $FADH_2$ in CBQ must be responsible for this inhibition. However, $FADH_2$ in CBQ is not the direct substrate for LiP because the inhibition of LiP activity by CBQ is non-competitive. The low K_i value for CBQ also supports this idea. This suggests that the CBQ redox-cycle is not directly coupled to the LiP redox-cycle. However, as $FADH_2$ in CBQ is effectively oxidized by LiP in the presence of veratryl alcohol, the CBQ redox-cycle must indirectly couple with the LiP redox-cycle. Thus, we propose that, as shown in Fig. 4, veratryl alcohol plays a role as radical-mediator between the LiP and CBQ redox-cycles. The ability of CBQ to reduce the 1,2,4,5-tetramethoxybenzene cation radical also supports this idea.

The positive effects of veratryl alcohol on LiP activity has been reported in several papers. The presence of veratryl alcohol in the LiP system facilitated depolymerization of DHP [10], oxidation of 4-methoxymandelic acid, anisyl alcohol [11], pyrene [12] and 2-hydroxy-1,4-naphthoquinone [13] and decarboxylation of oxalic acid [14,15]. Two possible explanations have been proposed for the role of veratryl alcohol in the LiP system: either veratryl alcohol protects LiP from inactivation by excess H_2O_2 [12]; or it functions as a radical mediator [11]. Recently, after intensive investigation of the role of veratryl alcohol in the LiP system, Gold and co-workers concluded that the major role of veratryl alcohol is to prevent inactivation of LiP by excess H_2O_2 [16,17]. However, recent reports suggest that veratryl alcohol acts as a radical mediator in the decarboxylation of oxalate by the LiP system [14,15] and in the electrochemical oxidation of the polymeric dye poly(B-411) by iron meso-tetra(2,6-dichloro-3-sulfonatophenyl)porphyrin chloride [18]. In this study, we have demonstrated that veratryl alcohol plays a radical-mediating role between the LiP and the CBQ systems. Thus, we conclude that veratryl alcohol not only prevents LiP inactivation by excess H_2O_2 but also plays a radical-mediating role.

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