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Is cellobiose oxidase from *Phanerochaete chrysosporium* a one-electron reductase?

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The heme domain of cellobiose oxidase (CBO) from *Phanerochaete chrysosporium* increases the rate of electron transfer to one-electron acceptors. This conclusion was drawn from comparisons of the rates of reduction of 3,5-*t*-butyl-*o*-benzoquinone, triiodide ion, cytochrome *c* and ferricyanide by intact CBO, FAD fragment and CBO with the heme inactivated by cyanide. The oxidation of cellobiose produced hydrogen peroxide, but the enzyme disturbs peroxidase-based assays by reduction of the product or by direct interaction with the peroxidase. CBO can also degrade hydrogen peroxide in the presence of cellobiose. The 1,2,4,5-tetramethoxybenzene cation radical was rapidly reduced by CBO.

Introduction

Cellobiose-oxidizing enzymes are produced under cellulolytic conditions by various white rot [1–6] and brown rot [7] fungi. Most studied are cellobiose oxidase (CBO) and cellobiose-quinone dehydrogenase (CBQ) from *Phanerochaete chrysosporium* and cellobiose dehydrogenase (GGDH) from *Thielavia heterothallica* (*Sporotrichum thermophile*). CBQ contains an FAD [1], whereas CBO/GGDH contain both an FAD and a heme group [2,7]. CBQ is probably a natural fragment derived from CBO by proteolysis [9,11]. These enzymes oxidize cellobiose and related oligosacharides (not glucose) to the corresponding lactones [1,2,7]. At least CBO and CBQ show strong adsorption to cellulose [8,9], but it is not quite clear whether the polysaccharide can serve as an electron donor [2,10], although a recent publication indicates this [12]. To our knowledge it is not known whether GGDH and other cellobiose oxidizing enzymes bind to cellulose. A large group of substances can serve as electron acceptors: molecular oxygen [2,9], quinones [1,9], triiodide ion [13], dichlorophenol indophenol [10], ferricyanide [7], ferriacetate [14] and cytochrome *c* [7,15].

The true biological function of these enzymes is not known, but the list of suggestions is impressive: oxidation of the reducing ends of cellulose chains [2], diminution of the product inhibition of cellulases [2], production of hydrogen peroxide [9] or superoxide anion [10], reduction of radical intermediates in the breakdown of lignin, such as the veratryl alcohol cation radical [15,16], bacteriocidal activity [17], detoxification [18] and reduction of Fe^{3+} in a Fenton type reaction [14].

There has been considerable confusion about the primary product of oxygen reduction by CBO, especially concerning hydrogen peroxide. Experimental data depend on the method employed for detection of hydrogen peroxide: catalase/oxygen electrode methods have indeed detected hydrogen peroxide in significant yields, whereas methods based on peroxidase and chromophoric substrate have given negative results [2,7,19], suggesting that CBO interferes with the peroxidase assay, e.g., by rapid electron transfer to the product of the peroxidase reaction.

Studies of the isolated heme and FAD domains, respectively, show that both the reductive and the oxidative half-reactions take place on the FAD domain, at least with oxygen or quinones as electron acceptors. The FAD domain is also responsible for the

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Abbreviations: ABTS, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; CBO, cellobiose oxidase (*P. chrysosporium*); CBQ, cellobiose-quinone dehydrogenase (*P. chrysosporium*); GGDH, cellobiose dehydrogenase (*T. heterothallica*); TMB, 1,2,4,5-tetramethoxybenzene.

Enzyme: cellobiose oxidase (EC 1.1.99.18).

binding to cellulose [9]. The real function of the heme domain, which may be the clue to understanding the function of the whole enzyme, is thus unknown, but the fact that it is rapidly reduced in the presence of cellobiose [2,10] and comparisons with the more studied flavocytochrome enzymes [20] suggest that it helps to transfer electrons from a two-electron donor such as cellobiose to one-electron acceptors such as cytochromes, ferric ion [14] or organic radicals produced during lignin breakdown [15,21]. In this work we compare the action of intact CBO with that of the isolated FAD fragment to reveal qualitative or quantitative features associated with the heme domain. The results form the basis for a suggested electron transfer mechanism.

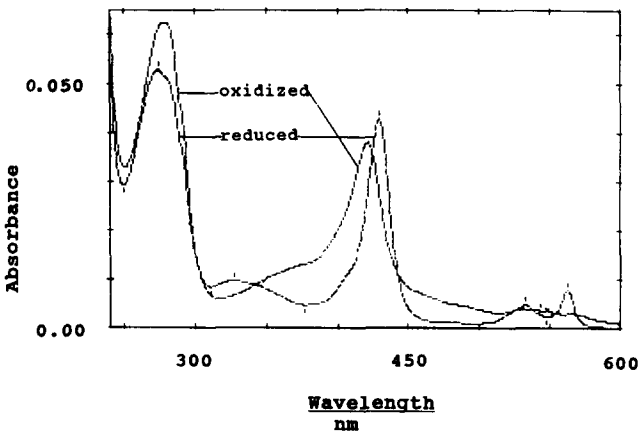

Fig. 1. Absorbance spectra of CBO before and after addition of cellobiose.

Materials and Methods

Materials

Cytochrome *c* type III from horse heart, superoxide dismutase from horseradish peroxidase type II from horseradish and 2,2-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were from Sigma (USA). The 1,2,4,5-tetramethoxybenzene (TMB) was a kind gift from Dr Paul Ander, Swedish University of Agicultural Sciences, Uppsala. Other chemicals were of analytical grade. Ultraviolet/visible spectra were recorded on a Shimadzu UV-160A spectrophotometer.

Enzyme production and purification

P. chrysosporium strain K 3 was grown in a fermenter and initial downstream procedures were carried out as described in Ref. 22. CBO was purified and the FAD fragment was prepared by limited proteolysis as described in Ref. 9.

Kinetic measurements

The rates of reduction of 3,5 di-*t*-butyl-*o*-benzoquinone, cytochrome *c*, ferricyanide and triiodide ion by CBO, FAD fragment [9] and CBO with the heme inactivated by cyanide ion, were determined spectrophotometrically at a constant temperature of 37°C. The wavelength used was 420 nm for ferricyanide and the 3,5-*t*-butyl-*o*-benzoquinone, 415 nm for cytochrome *c* and 350 nm for I_3^-. The difference in absorbance between reduced and oxidized state of cytochrome *c* is 33700 $M^{-1}cm^{-1}$ [23]. Extinction coefficient used was 1040 $M^{-1}cm^{-1}$ for $Fe(CN)_6$ [14], 26200 $M^{-1}cm^{-1}$ for triiodide [13], and 812 $M^{-1}cm^{-1}$ for the quinone. The experiments were performed at pH 5.0 using 0.2 mM cellobiose. K_m and k_{cat} were calculated from Lineweaver-Burk plots ($v^{-1} = f([S]^{-1})$) and the direct linear plot method [24]. The same stock solutions of the enzymes were used in all experiments.

Cyanide inactivation of the heme group was carried out as follows: 100 μl of 3.0 μM CBO in 75 mM ammonium acetate (pH 5.0) was mixed with appropriate volumes of 0.5 M KCN and adjusted to a final volume of 500 μl with 75 mM ammonium acetate (pH 5.0). The pH of the solutions stays within the activity range of the enzyme.

Spectra from 240 to 600 nm were recorded. 50 μl 40 mM cellobiose was then added and the spectrum was recorded again to reveal reduction of the heme group [2,10] (Fig. 1). Reduction rates of ferricyanide and 3,5 di-*t*-butyl-*o*-benzoquinone were determined as above with an excess of electron acceptor.

Determination of hydrogen peroxide

The production of hydrogen peroxide was monitored as follows: CBO or FAD fragment, cellobiose and, in some cases, hydrogen peroxide were mixed to appropriate concentrations in 50 mM sodium acetate, pH 5.0. The mixtures were incubated at 30°C for different times, and then boiled for two minutes to inactivate CBO. A 600 μl aliquot was analyzed for cellobiose using dinitrosalicylic acid reagent [25]. Another 30 μl-aliquot was mixed with 350 μl 0.1% ABTS, 10 μl 0.3 μM horseradish peroxidase and 610 μl 50 mM sodium acetate (pH 5.0). Hydrogen peroxide was determined from the resulting absorbance at 725 nm. Standards of hydrogen peroxide and cellobiose were incubated in parallel with the samples.

Experiments with ABTS as electron acceptor were performed as follows: 20 μl 0.3 μM peroxidase, 60 μl 0.1% ABTS, 100 μl 4 mM H_2O_2, 100 μl 90 mM cellobiose and 1000 μl 50 mM sodium acetate (pH 5.0) were mixed in a cuvette. After incubation for 1 min at room temperature the absorbance was measured at 725 nm and 10 μl 3 μM CBO in 50 mM ammonium acetate (pH 5) was added. The disappearance of oxidized ABTS was monitored at 725 nm. When the absorbance had stabilized a final spectrum was recorded.

Reduction of horseradish peroxidase

400 μl 4 mg/ml horseradish peroxidase in 75 mM ammonium acetate (pH 5) was mixed with 20 μl of 15 μM CBO in the same buffer. The spectrum from 240 to 600 nm was recorded. The peroxidase was oxidized by 7 μl 9 mM H_2O_2, and the spectrum was recorded again. 12.5 μl 40 mM cellobiose was added and the time course of the peroxidase reduction was monitored at 403 nm. When the absorbance had stabilized a final spectrum was recorded.

Reduction of 1,2,4,5-tetramethoxybenzene

1,2,4,5-Tetramethoxybenzene (TMB), hydrogen peroxide and cellobiose were mixed in a cuvette to final concentrations of 1 mM, 0.08 mM and 0.4 mM, respectively. Solutions of CBO or FAD fragment containing horseradish peroxidase were prepared and added to the substrate solutions giving final concentrations of 55 nM (CBO and FAD fragment) and 0.04 mg/ml (peroxidase). The peroxidase oxidizes the TMB to the corresponding cation radical which has a strong absorbance at 450 nm [15]. The change in absorbance at 450 nm due to reduction of the TMB cation radical was measured. We established that the cellobiose oxidizing enzymes had the same activity in the standard assay [9]. A blank experiment with only horseradish peroxidase was also performed.

Results

Both the intact CBO and the FAD fragment of CBO can use cytochrome *c* and ferricyanide as electron acceptors. Although intact CBO shows a significantly higher k_{cat} for the one-electron acceptors (cytochrome *c* and ferricyanide) than does the FAD fragment, they have similar values for the two-electron acceptors investigated (triiodide ion and 3,5-di-*t*-butyl-*o*-benzoquinone) (Table I) [9]. On the other hand, the differences in the K_m values are relatively small. The reduction of cytochrome *c* was not influenced by the presence of superoxide dismutase. Obviously, this reduction cannot be an effect of superoxide anion.

The reduction of the heme group using cellobiose as electron donor was strongly inhibited by a total KCN concentration higher than 15 mM. The presence of 100 mM KCN did not influence the CBO activity measured in the standard assay with 3,5-di-*t*butyl-*o*-benzoquinone as electron acceptor [9], whereas the rate of ferricyanide reduction by CBO was reduced by half under the same conditions.

CBO formed hydrogen peroxide when incubated with cellobiose as shown in Table II and Fig. 2. However, the amount of hydrogen peroxide produced is lower than the amount of cellobiose consumed. Furthermore, when hydrogen peroxide was present from the start, the concentration decreased in the presence of CBO and cellobiose. In a control experiment without cellobiose the effect was small (Table II and Fig. 3). Similar results were obtained with the FAD frag-

TABLE I

Michaelis constants and k_{cat} values for one-and two-electron acceptors of CBO and FAD fragment

Values determined from Lineweaver-Burk plots. The direct linear plot method [24] gave similar results. The concentration of the electron donor (cellobiose) was 0.2 mM in all experiments. The measurements were made at 37°C and at pH 5.

Electron acceptor	Enzyme	K_M (μM)	k_{cat} (s^{-1})	k_{cat}(CBO)/ k_{cat}(FAD fragm.)
Quinone	CBO	$5 \cdot 10^2$	17	1
	FAD fragm.	$4 \cdot 10^2$	15	
I_3^-	CBO	0.2	17	1
	FAD fragm.	0.3	14	
$Fe(CN)_6^{3-}$	CBO	$7 \cdot 10^3$	5.5	5
	FAD fragm.	$4 \cdot 10^3$	1.0	
Cytochrome *c*	CBO	0.3	13	$2 \cdot 10^2$
	FAD fragm.	0.3?	0.07	

TABLE II

Concentration of hydrogen peroxide and cellobiose after incubation with CBO

1 ml samples with 0.3 μM CBO were incubated in screw cap tubes at 30°C for 26 h. Concentrations of hydrogen peroxide and cellobiose were determined as described in the text.

Initial [cellobiose] (mM)	$[H_2O_2]$ (mM)	Remaining [cellobiose] (mM)	$[H_2O_2]$ (mM)
0.50	0	< 0.2	0.06
1.00	0	< 0.2	0.006
1.50	0	0.3	0.01
1.50	1.0	0.5	0.06
1.50	1.5	0.4	0.07
1.50	2.0	0.8	0.2
0	1.0	0	1.0
0	1.5	0	1.5
0	2.0	0	2.1

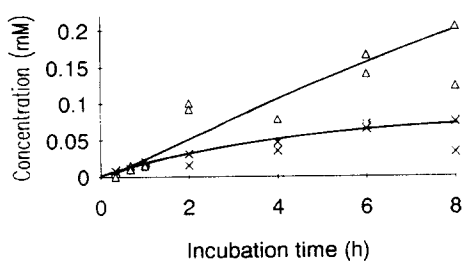

Fig. 2. Formation of hydrogen peroxide and consumption of cellobiose by CBO. Hydrogen peroxide formed (×). Cellobiose consumed (△). The experiment was carried out at 30°C as described in the text.

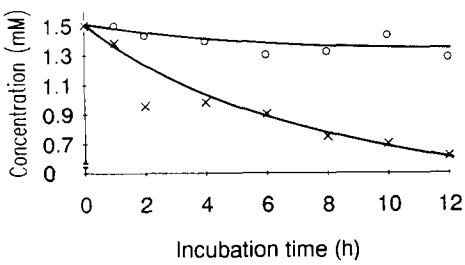

Fig. 3. Degradation of hydrogen peroxide by CBO. Hydrogen peroxide incubated with CBO and cellobiose (×). Hydrogen peroxide incubated with CBO (○). The experiment was performed as described in the text.

TABLE III

Concentration of hydrogen peroxide and cellobiose after incubation with CBO or FAD fragment

1 ml samples with 0.2 μM enzyme with the same activity in the standard assay [9], were incubated in screw cap tubes at 30°C for 27 h. Concentrations of hydrogen peroxide and cellobiose were determined as described in the text.

Enzyme	Initial [cellobiose] (mM)	$[H_2O_2]$ (mM)	Remaining [cellobiose] (mM)	$[H_2O_2]$ (mM)
FAD fragm.	1.50	0	1.21	0.18
CBO	1.50	0	0.80	0.21
FAD fragm.	1.50	1.50	1.12	1.46
CBO	1.50	1.50	0.93	0.91

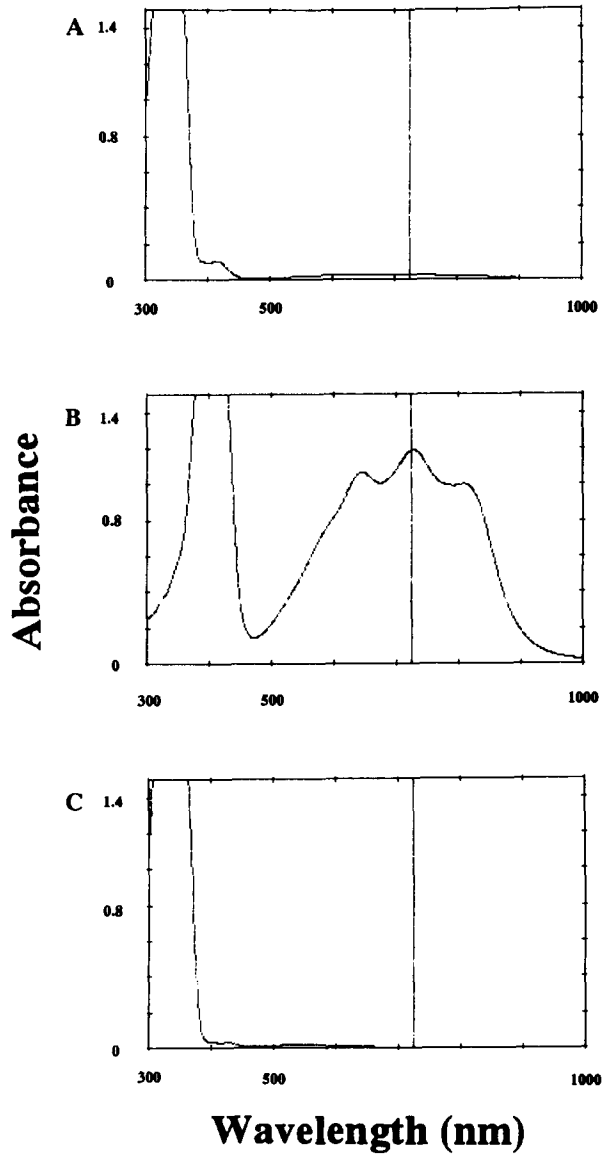

Fig. 4. Reduction of ABTS by CBO. Absorbance spectra of ABTS: (a) initial state (reduced). (b) After oxidation by horseradish peroxidase and hydrogen peroxide. (c) After oxidation followed by reaction with CBO and cellobiose. 725 nm is marked by a bar.

ment, although the ability to degrade hydrogen peroxide was less pronounced (Table III).

The expected green color was developed when hydrogen peroxide, cellobiose, peroxidase and the ABTS were mixed, and a peak at 725 nm was obtained. When CBO was added the color disappeared rapidly in parallel with the decrease in absorbance at 725 nm and the absorbance spectrum finally returned to the original state (Fig. 4). In an experiment without ABTS, the absorbance of horseradish peroxidase was monitored at 403 nm. On addition of H_2O_2, the absorbance decreased rapidly to a new stable value concomitantly with a shift of maximal absorption from 403 nm to 418 nm (probably Compound II). When cellobiose was added, the absorbance at 403 nm increased at an initial rate of 0.9 unit/min. After 10 min the absorbance became stable again and the spectrum showed that the peroxidase had returned to the initial state (Fig. 5). Obviously, it is impossible to determine hydrogen peroxide with peroxidase and ABTS if active CBO is present.

CBO and the FAD fragment do reduce the TMB cation radical faster than the spontaneous decay (Fig. 6). However, CBO reduces the radical approximately twice as fast as does the FAD fragment.

Discussion

Hydrogen peroxide is produced when electrons are transferred from cellobiose to oxygen by CBO or the isolated FAD-fragment, but considerably less than the expected 1:1 stoichiometry in both cases, and the relation was not linear (Tables II, III; Fig. 2). Similar experiments with hydrogen peroxide initially present even showed a net degradation of hydrogen peroxide. If a one-electron reduction is involved here, OH^- and $OH\cdot$ are formed. The latter, a vigorously reactive component, can attack both cellulose and lignin. Thus,

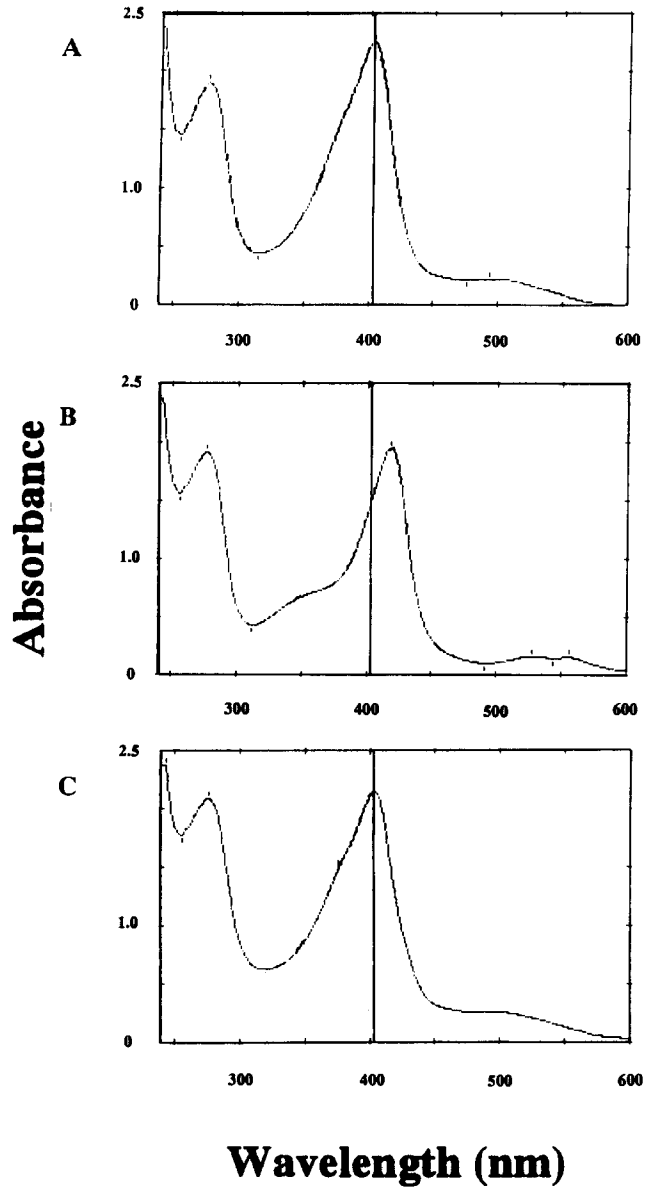

Fig. 5. Reduction of horseradish peroxidase by CBO. Absorbance spectra of Horseradish peroxidase: (a) Relaxed state. (b) After oxidation by hydrogen peroxide. (c) After reaction with CBO and cellobiose. 405 nm is marked by a bar.

CBO may act as a Fenton reagent also in the absence of free Fe^{3+}.

The question concerning hydrogen peroxide production is also complicated by the fact that CBO can readily use the oxidized forms of not only the peroxidase substrate, ABTS, but also horseradish peroxidase itself as electron acceptors (Figs. 4, 5), thus virtually 'destroying the evidence' for hydrogen peroxide production and giving a negative result [2,7,19]. A similar interaction with peroxidases may influence the lignolytic process [26].

It has so far been impossible to assign the heme domain a specific function, since virtually all reactions that are catalyzed by CBO also can be handled by the flavin domain, [9]. The results presented in Table I show, however, considerable *quantitative* decreases in the k_{cat} for one-electron acceptors (cytochrome *c* and ferricyanide) when the heme domain is removed whereas the change in K_m is small. These results agree with other recent observations that CBO reduces cytochrome *c* at a higher rate than CBQ does [15]. The reduction rates for two-electron acceptors, on the other hand, are virtually unchanged. The effect of cyanide also suggests a quantitative rather than specific role of the heme domain.

There are at least two possible models for this action (Fig. 7). In our opinion, model II, where in the FAD directly interacts with the electron acceptor, is most probable, since the isolated FAD fragment can indeed reduce one-electron acceptors. Here the heme functions as a reversible electron sink, allowing the enzyme to avoid the semiquinone state, which often reacts slowly with one-electron acceptors [27]. Such a mechanism is known for xanthine oxidase [27], whereas the yeast flavocytochrome b_2 works essentially according to model I [20]. The fact that the heme becomes reduced by an excess of cellobiose and in absence of electron acceptor (Fig. 1) does not exclude model II; it can be explained by interaction between different CBO molecules [28].

Both models may actually be valid, with the reduction of TMB cation radical and ferricyanide following path II and the reduction of cytochrome *c* mainly following path I [15].

Among the *natural* candidates for the one-electron acceptor 'A' we find O_2, giving reactive superoxide anion [7,10,29], or hydrogen peroxide, giving the still more reactive hydroxyl radical, which can degrade both cellulose and/or lignin. Here it should be noted that the FAD fragment degrades hydrogen peroxide slower than does CBO. The cellulose binding site of CBO [8,9]

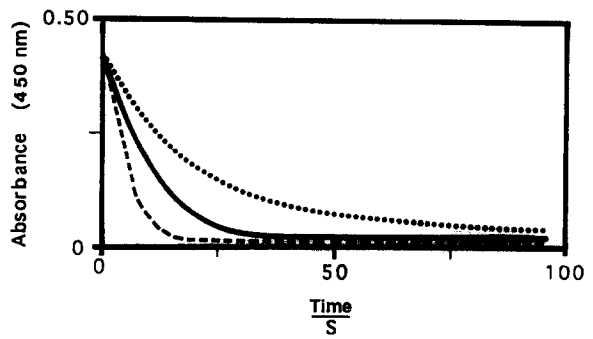

Fig. 6. Reduction of the TMB radical by CBO. Spontaneous decay (·····) of the TMB cation radical compared with reduction by CBO and cellobiose (— — —), and by FAD fragment and cellobiose (———). The experiment was carried out as described in the text.

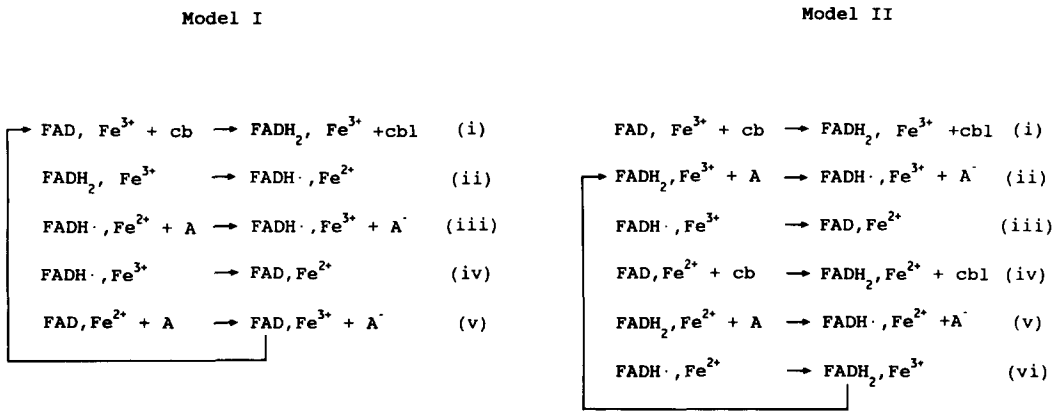

Fig. 7. Two models for the reduction of one-electron acceptors with CBO. 'FAD' and 'Fe' represent the flavin- and the heme prostetic groups of CBO, respectively. 'cb' is cellobiose and 'cbl' is cellobionolactone. 'A' is a one-electron acceptor, such as cytochrome *c*, $Fe(CN)_6^{3-}$ or O_2. The FAD oxidizes the cellobiose in both models. In model I, 'the electron transport chain model', an electron is transported to the one-electron acceptor by the heme group. In model II, 'the electron sink model', the electron is transported by the FAD and the heme group serves as an 'electron sink' that keeps the FAD in the fully reduced or oxidized form.

makes the diffusion distance for the radical to the cellulose very short.

Other possible electron acceptors are aromatic radicals formed by the action of lignin peroxidases [15], which may lose their tendency to repolymerize if they are reduced. The TMB cation radical, a well known model substance [21], is reduced efficiently by CBO and, although at a lower rate, by the FAD fragment (Fig. 6). Similar results have been reported for CBQ [21], which is suggested to be a natural FAD fragment [9,11], and recently also for CBO [15,30].

These data suggest that CBO, immobilized on the cellulose [8,9] may prevent undesirable de novo synthesis of lignin from organic cation radicals on the naked cellulose [30].

Complexed ferric ion has also been suggested as a natural electron acceptor [14], since it, together with hydrogen peroxide, constitutes a Fenton reagent. We want to recall, however, that CBO shows a Fenton-type activity without supplementary ferric ions.

In conclusion, our data suggest that CBO transfers electrons to some still not identified one-electron acceptor with cellobiose serving as a two-electron donor and with the heme group in a rate-enhancing role.

Acknowledgements

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