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ORIGINAL ARTICLE

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In vitro reduction of manganese dioxide by a ferrireductase system from the white-rot fungus *Phanerochaete sordida* YK-624

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Abstract Reduction of manganese dioxide is demonstrated for an in vitro ferrireductase system that includes NADPH-dependent ferrireductase and the iron-binding compound (IBC) isolated from the white-rot fungus *Phanerochaete sordida* YK-624. The Fe(II)–IBC complex was more effective in reducing manganese dioxide to Mn(II) than were complexes of Fe(II) and organic acids of low molecular weight such as nitrilotriacetate, although IBC also reduced manganese dioxide to Mn(II) in the absence of Fe(II). The generated Fe(III)–IBC complex was a better substrate for NADPH-dependent ferrireductase than were other ferric chelates, suggesting that the Fe(III)–IBC complex is reduced to an Fe(II) complex by NADPH-dependent ferrireductase. Moreover, production of the Fe(III)–IBC complex by the reduction of manganese dioxide in a reaction system containing Fe(II) and IBC was observed to be coupled to reduction of the Fe(III)–IBC complex by NADPH-dependent ferrireductase. These results indicate that the ferrireductase system of *P. sordida* YK-624 plays an important role in the reduction of manganese dioxide, which is necessary for the production and function of manganese peroxidase.

Key words *Phanerochaete sordida* YK-624 · NADPH-dependent ferrireductase · Iron-binding compound · Manganese dioxide · In vitro reduction

Introduction

Several species of white-rot fungi have been studied intensively in recent years because of their ability to degrade and remove lignin from wood. In response to environmental concerns and increasingly stringent emissions standards, the pulp and paper industry has been looking for ways to decrease the levels of chlorinated lignin residues in its effluents through production process changes and improved treatment technologies. The white-rot fungi *Phanerochaete chrysosporium*,[1,2] *Trametes versicolor*,[3,4] *P. sordida* YK-624,[5,6] and IZU-154[7,8] have the ability to bleach kraft pulp. Manganese peroxidase (MnP) is a key enzyme in the biological bleaching of kraft pulp with white-rot fungi because the MnP activity levels detected in cultures have been found to be linearly correlated with the increase in brightness of the kraft pulp treated with these fungi.[1,5,9]

The Mn(II) ion is necessary for the production and function of MnP from *P. sordida* YK-624 and other fungi.[6] Unbleached hardwood kraft pulp (HWKP) contains Mn at a concentration of about 50 mg/kg pulp.[6,9] That *P. sordida* YK-624 uses manganese from HWKP in its biological bleaching is evident from the observation that this fungus can produce MnP and brighten HWKP in cultures containing only HWKP and water.[5,6] Treatment with MnP alone does not increase the brightness of HWKP without the addition of $MnSO_4$,[10] but if oxalate is added as a manganese dioxide-reducing agent, MnP can brighten HWKP in vitro without exogenous $MnSO_4$.[11] These results suggest that the manganese in HWKP occurs predominantly in the Mn(IV) oxidation state in the form of compounds such as manganese dioxide and that little Mn(II) is present. As part of the process of biological bleaching, *P. sordida* YK-624 apparently reduces manganese dioxide in HWKP to Mn(II). Electron spin resonance analysis of HWKP biobleaching with *P. sordida* YK-624 has confirmed that manganese dioxide [Mn(IV)] in kraft pulp is reduced to Mn(II).[12,13] The reduction of manganese triggers *P. sordida* YK-624 to produce MnP, which then bleaches the kraft pulp.[12]

Washed mycelia of *P. sordida* YK-624 in liquid culture have been observed to reduce manganese dioxide,[13,14] a process demonstrated to be dependent on Fe(II), a metal chelator, and NAD(P)H. Moreover, cell-free extracts of *P. sordida* YK-624 have been demonstrated to catalyze the reduction of ferric chelate to ferrous chelate in the presence of NAD(P)H.[13] It is likely, therefore, that ferrous chelate reduces manganese dioxide and that the ferric chelate pro-

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duced is reduced, in turn, by an intracellular NAD(P)H-dependent reductase to regenerate the ferrous chelate.[13,14] An intracellular NADPH-dependent ferrireductase (molecular weight 35 kDa; pI 5.1) has been purified from *P. sordida* YK-624.[13,15] It is necessary for the reduction of Fe(III)–nitrilotriacetate (NTA) complex by the ferrireductase to add 1,10-phenanthroline (PHT) as an effective ferrous chelator to the reaction mixture.[15] This result suggests that the Fe(III)–NTA complex is not a good substrate for the ferrireductase, and that PHT may play an important role in the release of Fe(II) from the ferrireductase. In short, it is probable that *P. sordida* YK-624 produces IBC as iron chelator and that Fe(III)–IBC is directly reduced by the ferrireductase. More recently, we have isolated IBC from a culture of *P. sordida* YK-624.[16] IBC is probably involved in the reduction of manganese dioxide by combination with Fe(II), in the transport of iron into cells, and in the reduction of Fe(III) by the ferrireductase as chelator.

Here we describe the in vitro reduction of manganese dioxide by a ferrireductase system that includes NADPH-dependent ferrireductase, an IBC, Fe(II), and NADPH. Particularly, we examined whether Fe(II)–IBC complex is a good electron donor for the reduction of manganese dioxide, and whether Fe(III)–IBC complex is a good substrate for the ferrireductase.

Materials and methods

Microorganism

Fungus strain *P. sordida* YK-624 (ATCC 90872) was used in this study. The strain was maintained on potato dextrose agar (PDA; Difco) slants at 4°C.

Production and purification of NADPH-dependent ferrireductase

The activity of NADPH-dependent ferrireductase was determined as described previously.[15] PDA plates (9 cm diameter) were inoculated with the strain and incubated for 3 days at 30°C. Four disks punched from the growing edge of the mycelium on a PDA plate were added to a petri dish (9 cm diameter) containing 20 ml of a liquid medium and statically incubated at 30°C for 7 days. The liquid medium contained 1% glucose, 12 mM ammonium tartrate, 20 mM sodium acetate, 1% Tween 80, 2 mM vanillic acid, 14.7 mM KH_2PO_4, 2.16 mM NTA, 2.80 mM $MgSO_4$, 1.72 mM $MnSO_4$, 6.33 mM NaCl, 0.24 mM $CoSO_4$, 1.14 mM $CaCl_2$, 0.24 mM $ZnSO_4$, 14.8 μM $CuSO_4$, 14.3 μM $AlK(SO_4)_2$, 60.7 μM H_3BO_3, 17.8 μM Na_2MoO_4, and 3 μM thiamine-HCl, adjusted to pH 4.5. Fifty petri dishes were used.

Enzyme extraction was carried out as reported previously.[15] The extract was fractionated by sequential additions of solid ammonium sulfate. Proteins exhibiting ferrireductase activity precipitated at 30%–65% ammonium sulfate saturation and were redissolved in 20 mM sodium phosphate (pH 7.0). A column (1.6 × 10 cm) of HiPrep 16/10 Butyl FF (Pharmacia Biotech, Upsala, Sweden) was equilibrated with 20 mM sodium phosphate (pH 7.0) containing 1 M ammonium sulfate. The protein was applied in the equilibration column, and the column was washed with 100 ml equilibration buffer. The protein was then eluted with a linear gradient of decreasing ammonium sulfate concentration (1 M to 0 M; total volume 120 ml). The fractions containing NADPH-dependent ferrireductase activity were pooled and concentrated by ultrafiltration (10 kDa cutoff).

The active fraction from the HiPrep 16/10 Butyl FF column was applied to a column (2.0 × 50 cm) of HiLoad 16/60 Superdex 75 pg (Pharmacia Biotech) equilibrated at a flow rate of 0.5 ml/min with 20 mM sodium phosphate (pH 7.0) containing 0.1 M ammonium sulfate. Active fractions from the Superdex 75 pg column were desalted by passage through a PD-10 (Pharmacia Biotech) column equilibrated with 10 mM borate buffer (pH 9.3) and concentrated by ultrafiltration (10 kDa cutoff). The concentrated solution was loaded onto a Mono Q HR 5/5 (Pharmacia Biotech) column equilibrated with 10 mM borate buffer (pH 9.3). Unbound protein was washed off with 15 ml of 10 mM borate buffer (pH 9.3), and the active fractions were eluted with a linear gradient of increasing ammonium sulfate concentration (0–0.5 M) at a flow rate of 0.5 ml/min. The active fractions eluted from the Mono Q column were used in the present study.

Production and purification of IBC

Iron-binding activity was confirmed by the CAS universal chemical assay.[17] Four disks punched from the growing edge of the mycelium on a PDA plate were added to a petri dish (9 cm diameter) containing 20 ml of a liquid medium and statically incubated at 30°C for 6 days. The liquid medium contained 1% glucose, 12 mM ammonium tartrate, 20 mM sodium acetate, 14.7 mM KCl, 2 mM $MgSO_4$, 0.88 mM $CaCl_2$, and 3 μM thiamine HCl, adjusted to pH 4.5. Mycelial mats were separated from the cultures, and the culture fluid was filtered with a 47-mm nitrocellulose membrane filter with 0.45 μm pore size (Toyo Roshi, Tokyo, Japan). The filtrate was lyophilized and dissolved in distilled water. A 2-ml aliquot of the solution was loaded onto a column of Sephadex G-25 (1.6 × 60 cm) (Superfine; Pharmacia Biotech) and equilibrated with distilled water. Active fractions were used in the present study.

Reduction of manganese dioxide by Fe(II)–IBC complex

The reaction mixture (30 ml) contained 20 mM 2,2-dimethylsuccinate (pH 4.5 or 7.0), 0.1 mM $FeSO_4$, IBC sufficient to bind with 100 nmol Fe(III), and 69 μmol manganese dioxide. The reaction was carried out at 30°C on a rotary shaker (150 rpm) for 12 h. The reaction mixture then was filtered with a membrane filter (0.45 μm pore size), and the concentration of manganese [as soluble Mn(II)] in the filtrate was measured by atomic absorption analysis.

Reduction of Fe(III)–IBC complex by NADPH-dependent ferrireductase

Reduction of ferric chelate by NADPH-dependent ferrireductase was determined by monitoring the oxidation of NADPH at 340 nm. The reaction mixture (1 ml) containing 20 mM sodium phosphate (pH 7.0), 0.1 nkat NADPH-dependent ferrireductase, 0.1 mM $FeCl_3$, and IBC [sufficient to bind 3.3 μmol Fe(III)] was incubated at 30°C. The reaction was initiated by the addition of 0.1 mM NADPH. Nitrilotriacetate (NTA) 0.5 mM, citrate 2 mM, and oxalate 2 mM were used as other ferric chelators in the present study.

In vitro reduction of manganese dioxide by ferrireductase system

Reduction of manganese dioxide by the Fe(II)–IBC complex and NADPH-dependent ferrireductase was examined as follows. In the first step, the reaction mixture (30 ml) contained 20 mM 2,2-dimethylsuccinate (pH 4.5), 69 μmol manganese dioxide, 10 μM $FeSO_4$, and either enough IBC to bind 100 nmol Fe(III) or 12 μmol NTA [which is able to bind 100 nmol Fe(III) in the CAS assay]. The reaction was conducted in a 100-ml Erlenmeyer flask at 30°C on a rotary shaker (150 rpm) for 6 h. The reaction mixture was then filtered with a 0.45-μm pore size membrane filter, and 100 μl of the filtrate was added to the reaction mixture (1 ml) containing 20 mM sodium phosphate (pH 7.0) and 0.5 nkat NADPH-dependent ferrireductase. The reaction was initiated by the addition of 0.1 μmol NADPH at 30°C. The reduction of ferric chelates was evaluated by monitoring the oxidation of NADPH at 340 nm.

Results and discussion

Reduction of manganese dioxide by Fe(II)-IBC

In the present study, IBC was produced in several components-omitting medium described above, different from our previous report,[16] as $CuSO_4$, $AlK(SO_4)_2$, $CoSO_4$, 2,2-dimethylsuccinate, Tween 80, KH_2PO_4, and NTA inhibited in the determination of iron-binding activity using the CAS universal chemical assay. The amount of IBC produced in the present medium was almost the same as that in the previous medium,[16] and the elution time of IBC, which was produced in the present medium, on Sephadex G-25 chromatography was the same as that in our previous medium.[16] To demonstrate the extracellular reduction of manganese dioxide, the capacity of the Fe(II)–IBC complex to reduce manganese dioxide to Mn(II) was determined. If Fe(II) reduces manganese dioxide, the reaction must obey the equation

$$2\,\mathrm{Fe(II)} + \mathrm{MnO_2} + 4\,\mathrm{H^+} \rightarrow 2\,\mathrm{Fe(III)} + \mathrm{Mn(II)} + 2\mathrm{H_2O}$$

Therefore, the reduction of manganese dioxide to Mn(II) by Fe(II) depends on the pH, and the predicted result was observed (Table 1). Fe(II)-dependent generation of Mn(II) occurred more readily at pH 4.5 than at pH 7.0. The Fe(II)–IBC complex also reduced manganese dioxide more effectively than did Fe(II) alone (Table 1). In the reaction mixture that included the Fe(II)–IBC complex (at pH 4.5), 496 μM Mn(II) was detected (Table 1); however, if only 100 μM Fe(II) is present in the reaction mixture, only 50 μM Mn(II) can be generated. This apparent inconsistency suggests that the IBC also functions as an electron donor for the reduction of manganese dioxide. Therefore, the possibility that IBC reduces manganese dioxide in the absence of Fe(II) was examined. As Table 1 shows, the IBC did reduce manganese dioxide to Mn(II), although the amount of Mn(II) generated by the IBC in the absence of Fe(II) was lower. The IBC also reduced Fe(III) and 2-methoxy-1,4-benzoquinone (data not shown). These results indicate that the IBC functions not only as a ferric chelator but also as an electron donor. Tanaka et al.[18] and Goodell et al.[19] have also isolated IBCs from several wood-rot fungi, and these compounds were able to reduce molecular oxygen and ferric iron. Although the structure of the IBC from *P. sordida* YK-624[16] has not yet been identified, it is thought to reduce Fe(III) and benzoquinones by a mechanism similar to that for those compounds from other wood-rot fungi.[18,19]

Table 1. Reduction of manganese dioxide by Fe(II)–IBC complex (12 h)

Substances	Mn(II) (μM)	
	pH 4.5	pH 7.0
Fe(II) + IBC + MnO_2	496	58
IBC + MnO_2	380	11
Fe(II) + MnO_2	16	2
IBC	1	2
MnO_2	1	0

Reduction of Fe(III)–IBC complex by NADPH-dependent ferrireductase

In our previous report,[15] NADPH-dependent ferrireductase hardly reduced Fe(III)–NTA complex without PHT. In short, NTA is an exogenous chelator, not an endogenous compound. More recently, endogenous IBC was isolated from *P. sordida* YK-624.[16] To demonstrate that NADPH-dependent ferrireductase reduces intracellular ferrous chelates, the Fe(III)–IBC complex and Fe(III)–organic acid complexes were compared with respect to their abilities to serve as substrates for NADPH-dependent ferrireductase. As shown in Fig. 1, the maximum rates of NADPH oxidation by NADPH-dependent ferrireductase were obtained in reaction mixtures containing the Fe(III)–IBC complex. NADPH oxidation by NADPH-dependent ferrireductase hardly occurred in the presence of Fe(III)–organic acid complexes such as Fe(III)–NTA, Fe(III)–citrate, and Fe(III)–oxalate. Moreover, oxidized IBC produced with 2-methoxy-1,4-benzoquinone did not affect the oxidation of NADPH by NADPH-dependent ferrireductase (data not

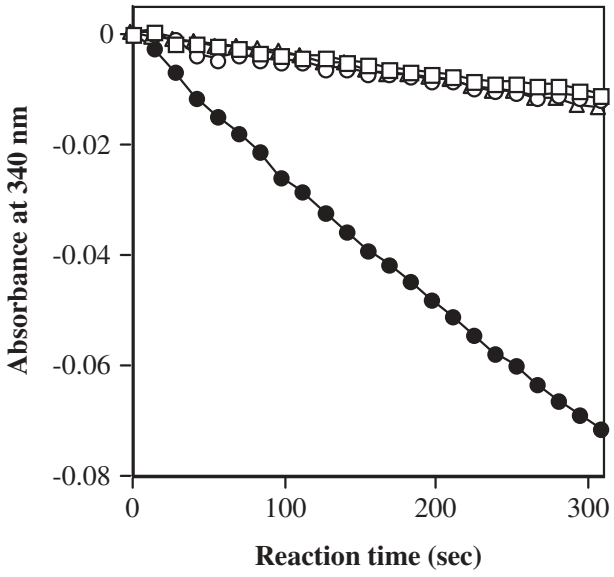

Fig. 1. Reduction of Fe(III) complexes of iron-binding compound (IBC) and organic acids by NADPH-dependent ferrireductase. These reactions were carried out at pH 7.0. *squares*, Fe(III)–NTA; *open circles*, Fe(III)–citrate; *triangles*, Fe(III)–oxalate; *filled circles*, Fe(III)–IBC

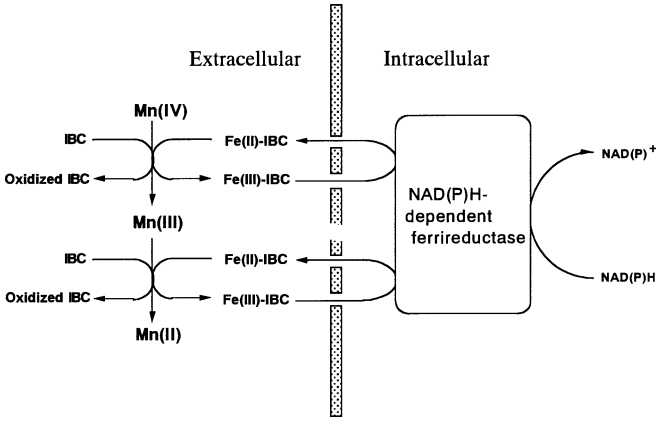

Fig. 2. Proposed mechanism of manganese dioxide reduction by the ferrireductase system of *P. sordida* YK-624

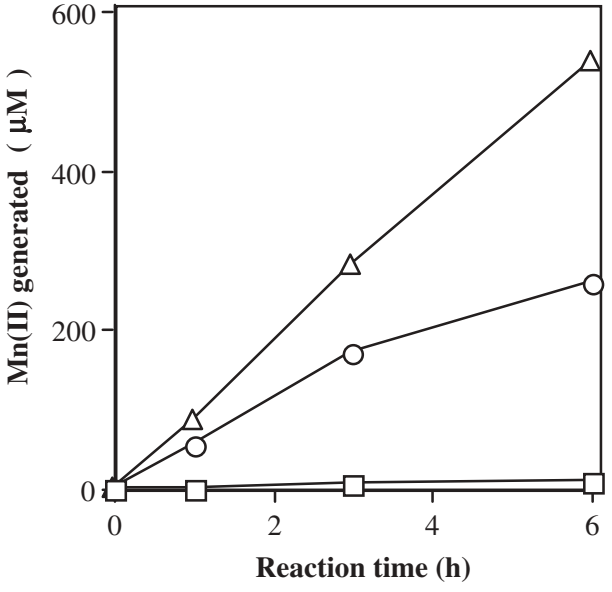

Fig. 3. Reduction of manganese dioxide by Fe(II)–IBC complex. *Squares*, Fe(II); *circles*, Fe(II)–NTA; *triangles*, Fe(II)–IBC

shown). These results suggest that Fe(III)–IBC complex is a good substrate for NADPH-dependent ferrireductase, and the enzyme reduces it to the Fe(II)–IBC complex. On the other hand, IBC was not a substrate for NADPH-dependent ferrireductase, although IBC reduced manganese dioxide to Mn(II) without Fe(II), as shown in Table 1. Therefore, Both Fe ion and IBC are necessary for the effective reduction of manganese dioxide by NADPH-dependent ferrireductase.

Reduction of manganese dioxide by the ferrireductase system

We then tried to demonstrate the in vitro reduction of manganese dioxide by a two-step ferrireductase system like the one presented in Fig. 2. The first step of the experiment, the reduction of manganese dioxide by Fe(II)–IBC complex, was carried out at pH 4.5 (i.e., under extracellular conditions). In the second step, NADPH-dependent ferrireductase was used to determine if NADPH is oxidized in a reaction mixture containing Fe(II)–IBC complex used in the first step of the experiment. Figure 3 demonstrates that the Fe(II)–IBC complex reduced manganese dioxide to Mn(II) at a higher rate than the Fe(II)–NTA complex, suggesting that the Fe(II)–IBC complex is a good electron donor for manganese dioxide compared to other ferrous chelates such as the Fe(II)–NTA complex. Moreover, NADPH-dependent ferrireductase efficiently oxidized NADPH in a reaction mixture containing manganese dioxide, Fe(II), and IBC used in the first step of the experiment (Fig. 4). In contrast, the reaction mixture containing NTA instead of IBC had little effect on NADPH oxidation by NADPH-dependent ferrireductase (Fig. 4). These results suggest that the Fe(II)–IBC complex is a good electron donor for manganese dioxide and that the Fe(III)–IBC complex is a substrate for NADPH-dependent ferrireductase.

Conclusions

Several findings of the present study demonstrated in vitro reduction of manganese dioxide by a ferrireductase system that includes NADPH-dependent ferrireductase and the IBC from *P. sordida* YK-624. First, the Fe(II)–IBC complex was a good electron donor for reduction of manganese dioxide, although IBC also reduced manganese dioxide to

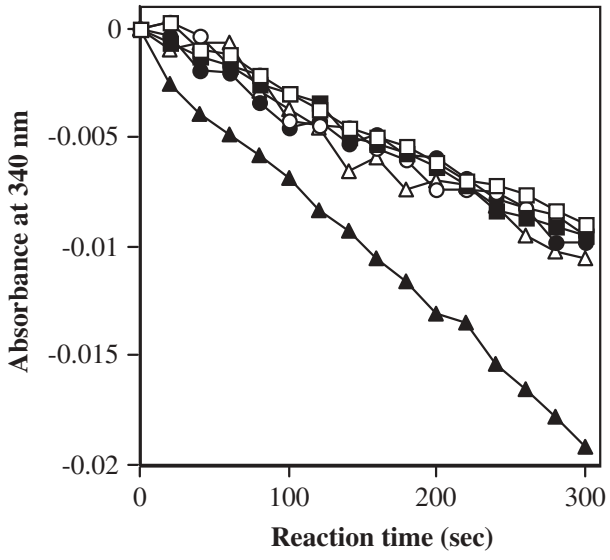

Fig. 4. Effect of the reaction mixture composition of the first step of the experiment on the oxidation of NADPH by NADPH-dependent ferrireductase. *Open squares*, with Fe(II) at 0 h incubation; *filled squares*, Fe(II) after 6 h of incubation; *open circles*, with Fe(II)–NTA at 0 h; *filled circles*, with Fe(II)–NTA after 6 h; *open triangles*, with Fe(II)–IBC at 0 h; *filled triangles*, Fe(II)–IBC after 6 h

Mn(II). Second, the Fe(III)–IBC complex was a ferric substrate of NADPH-dependent ferrireductase, but IBC was not. Finally, production of the Fe(III)–IBC complex by the reduction of manganese dioxide in a reaction system containing Fe(II) and IBC was coupled to the reduction of the Fe(III)–IBC complex by NADPH-dependent ferrireductase. These results strongly support the hypothesis that the reduction of manganese dioxide by *P. sordida* YK-624 is caused by the mechanism presented in Fig. 2, and that the ferrireductase system including IBC as both electron donor and iron chelator is necessary for the production and function of manganese peroxidase. The reducing mechanism of oxidized IBC by *P. sordida* YK-624 is being examined in detail and will be reported in a forthcoming paper.

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