Purification and partial characterization of an intracellular NADH:quinone oxidoreductase from *Phanerochaete chrysosporium*

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Phanerochaete chrysosporium produced several intracellular NADH:quinone oxidoreductases under agitated, nitrogen-limited cultivation conditions. One of the quinone reductases was purified and shown to have a molecular mass of 69 kDa by SDS-PAGE, while the molecular mass determined by gel filtration was 47 kDa. This reductase was separated by IEF into four protein bands, each with quinone reductase activity. The isoelectric points of the proteins were 5.7, 5.9, 6.0 and 6.3. The proteins reduced several quinones to the corresponding hydroquinones, but none of them was specific to any one of the quinones tested. Mycelial extracts of *P. chrysosporium* contained several more quinone reductases, with isoelectric points of 4.4, 4.7, 5.0, 5.3, 5.5 and 6.6. Quinone reductase activity could be induced by adding vanillic acid or 2-methoxy-1,4-benzoquinone to the growth medium in nitrogen-limited cultures and in carbon-limited cultures.

Introduction

The basidiomycete *Phanerochaete chrysosporium* produces several enzymes involved in the degradation of the plant cell wall biopolymer lignin (Tien & Kirk, 1983; Gold *et al.*, 1984). Among these enzymes, lignin peroxidases play a central role, but they are not the only factor necessary for the degradation of lignin to CO₂. *In vitro*, they polymerize rather than depolymerize lignin (Hämmerli *et al.*, 1986). Schoemaker *et al.* (1989) suggested that quinone metabolism might be one of these other factors, since various substituted quinones have been identified as key intermediates in the degradation of lignin model compounds (Ander *et al.*, 1980; Buswell & Eriksson, 1988; Higuchi, 1985; Schoemaker & Leisola, 1987; Schoemaker *et al.*, 1989). The rapid metabolism of quinones was proposed as a mechanism for shifting the equilibrium from enzymic polymerization to the depolymerization of lignin.

Several quinone reductases have been described which

could be responsible for catalysing the first step in the degradation of quinones. One of these reductases is dependent on cellobiose as co-substrate and is produced extracellularly (Westermarck & Eriksson, 1974*a,b*). The hypothesis that this reductase is involved in the degradation of lignin was supported by two observations (Ander *et al.*, 1990): firstly, the polymerization of kraft lignin by lignin peroxidase was decreased in the presence of this reductase; secondly, this reductase was shown to interact with laccase and different peroxidases, thereby increasing the decarboxylation of the lignin model compound vanillic acid. Another intracellular NAD(P)H:quinone oxidoreductase was described which reduces 2-methoxyquinone and several other synthetic quinones (Buswell *et al.*, 1979; Buswell & Eriksson, 1988). Later studies showed that *P. chrysosporium* produces at least two different intracellular quinone reductases. These may be part of a system which allows the fungus to take up small fragments of lignin and to degrade them intracellularly (Schoemaker *et al.*, 1989). Each of these intracellular quinone reductases was suggested to reduce only one of the different quinones produced by the enzymic oxidation of the lignin model compound veratryl alcohol (Fig. 1).

We describe here the purification and characterization of an intracellular quinone reductase from *P. chrysosporium* that uses 2-hydroxymethyl-5-methoxy-1,4-benzoquinone, 2-methoxy-1,4-benzoquinone and 2-methoxy-5-(methoxymethyl)-1,4-benzoquinone as sub-

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Abbreviations: IEF, isoelectric focusing; MTT tetrazolium, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide; QI, quinone I (2-hydroxymethyl-5-methoxy-2,5-cyclohexadiene-1,4-dione); QII, quinone II (2-methoxy-2,5-cyclohexadiene-1,4-dione); QIII, quinone III (4,5-dimethoxy-3,5-cyclohexadiene-1,2-dione); QIV, quinone IV (2-methoxy-5-(methoxymethyl)-2,5-cyclohexadiene).

Fig. 1. Structural formulae of the quinones used in this study.

strates, all of which are products of the oxidative degradation of veratryl alcohol or its methyl ether by lignin peroxidase (Schmidt *et al.*, 1989).

Methods

Chemicals. NADH and 2-methoxyhydroquinone were from Fluka. The quinones 2-hydroxymethyl-5-methoxy-2,5-cyclohexadiene-1,4-dione (QI) and 2-methoxy-2,5-cyclohexadiene-1,4-dione (QII) as well as 4,5-dimethoxy-3,5-cyclohexadiene-1,2-dione (QIII) and 2-methoxy-5-(methoxymethyl)-2,5-cyclohexadiene (QIV) were prepared according to Schmidt *et al.* (1989). The structures of QI–QIV are shown in Fig. 1.

The hydroquinones corresponding to QI and QIV were prepared according to Fieser & Fieser (1967) by the reduction of the quinones with $\text{Na}_2\text{S}_2\text{O}_4$.

Culture conditions. The white-rot fungus *Phanerochaete chrysosporium* (ATCC 24725) was maintained on 2% (w/v) malt agar slants at 4 °C. Twelve one-litre Erlenmeyer flasks were inoculated with 10^4 spores ml^{-1} . Each flask contained 600 ml N- or C-limited medium (Muheim *et al.*, 1990). The fungus was cultivated for 3.5 d on a rotary shaker (150 r.p.m.) at 37 °C.

Enzyme assay. Quinone reductase activity was measured in 25 mM-Tris/HCl, pH 8.0, with 250 μM -QI, II, III or IV and 200 μM -NADH. The decrease in absorbance at 340 nm due to NADH oxidation was monitored spectrophotometrically at 23 °C. NADH is also oxidized non-enzymically by the quinones. The rate of this reaction was measured in a blank containing no enzyme, and its value was subtracted from that of the sample containing enzyme in a dual-path spectrophotometer (Perkin Elmer). The rate of non-enzymic quinone reduction by DTT was also subtracted, when this compound was tested as an inhibitor of the quinone reductases.

One unit of enzyme was defined as the amount oxidizing 1 μmol NADH min^{-1} at 23 °C and pH 8.0. The NADH concentration was calculated using the equation $\Delta A = [(\epsilon_{\text{NADH}} + \Delta C_{\text{NADH}}) + (\epsilon_{\text{Q}} + \Delta C_{\text{Q}})] \times l$, where C = concentration and l = path length. Since 1 mol NADH reduces 1 mol quinone, ΔC_{NADH} is equal to ΔC_{Q} . Therefore $\Delta A = (\epsilon_{\text{NADH}} + \epsilon_{\text{Q}}) \times \Delta C \times l$. The absorption coefficients $\epsilon = \epsilon_{\text{NADH}} + \epsilon_{\text{Q}}$ obtained for QI, QII and QIV were 7.796, 7.374 and 7.027 $\text{mm}^{-1} \text{cm}^{-1}$, respectively.

The protein concentration was determined according to Bradford (1976).

Enzyme purification. The mycelial pellets were washed twice with distilled water and once with 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-EDTA. The pellets were suspended in 800 ml of the same buffer, homogenized for 5 min with a Polytron mixer (Kinematica, Luzern, Switzerland) and centrifuged at 40000 g for 20 min at 4 °C. CaCl_2 (4 M) and 50% (w/v) PEG were added to the supernatant to

give final concentrations of 16 mM and 10%, respectively. The solution was stirred for 30 min and then centrifuged at 40000 g for 20 min at 4 °C.

Swollen Blue Sepharose CL-6B gel (Pharmacia) (80 ml) was equilibrated with 25 mM-Tris/HCl, pH 7.5, containing 2 mM- CoCl_2 . The gel was added to 720 ml of the supernatant for the adsorption of quinone reductases and other nucleotide-dependent enzymes (Lowe & Pearson, 1984). After stirring for 15 min, the suspension was filtered through a sintered glass filter. The gel was packed on a column (32 mm diameter), which was then equilibrated with the same buffer. At a flow rate of 100 ml h^{-1} , quinone reductases were eluted with 25 mM-Tris/HCl, pH 8.0, containing 0.5 mM-NADH. Active fractions were pooled and concentrated to 11 ml by ultrafiltration.

The concentrate was applied to a Pharmacia Phenyl Superose HR 5/5 column (FPLC) equilibrated with 25 mM-Tris/HCl, pH 8.0. Proteins were eluted with a gradient from 1.7 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in a volume of 40 ml, using a flow rate of 0.5 ml min^{-1} . A sample of the eluate (0.6 ml) containing reductase activity was dialysed against 25 mM-Tris/HCl, pH 8.0, and applied to a Pharmacia Mono Q HR 5/5 column equilibrated with 25 mM-Tris/HCl, pH 8.0. The elution was performed at a flow rate of 1 ml min^{-1} with a gradient from 0 to 0.5 M-NaCl in a volume of 20 ml followed by a gradient from 0.5 to 1 M-NaCl in a volume of 10 ml.

Determination of molecular mass. The molecular mass of quinone reductases purified by Phenyl Superose was estimated by gel filtration on a Pharmacia Superose 12 column (FPLC). A 200 μl sample of the protein solution was applied to the gel filtration column which was equilibrated with 30 mM-Tris/HCl, pH 8.0, containing 50 mM-NaCl and 2% (v/v) glycerol. The flow rate was 0.5 ml min^{-1} . Protein standards from Pharmacia were used to calibrate the column.

In addition, SDS-PAGE was performed on a Phast System (Pharmacia) with 10–15% (w/v) polyacrylamide gradient gels. The gels were silver stained according to the Phast System protocol. The standard protein mixture was from Pharmacia.

Isoelectric focusing. Agarose IEF gels, pH 4–8, were prepared with 0.95 ml Bio-Lyte 4–6 and 0.95 ml Bio-Lyte 6–8. Isoelectric points were determined with standard proteins from Serva. IEF pH 4–6.5 for two-dimensional electrophoresis was performed on the Phast System according to the Phast System protocol.

Activity staining. In IEF gels the quinone reductases were activity-stained. The staining solution contained 25 mM-Tris/HCl, pH 8.0, 2 mM-MTT tetrazolium, 1 mM-NADH and 50 μM -QI, II or IV. The enzymically produced hydroquinones reduced MTT tetrazolium to MTT formazan, a violet insoluble dye (Höjeberg *et al.*, 1981).

Product analysis. The products of the enzymic reaction were analysed by HPLC (Merck-Hitachi System 655A-12). The reaction mixture contained 40 U quinone reductase ml^{-1} , 0.25 mM-quinone, 0.5 mM-NADH and 10 mM- NH_4HCO_3 , pH 7.8. Samples were taken at

Fig. 2. Time course of quinone reductase activity in crude extracts of N-limited cultures of *P. chrysosporium*, measured with QIV as the substrate. See Methods for details of the assay. The experiment was repeated three times; representative results are shown.

intervals of 15 min, and 20 μ l of the sample was loaded on a Nucleosil RP 18 column (Bischoff-Analysentechnik). The eluent (flow rate 1 ml min⁻¹) was a mixture of water and 20% (v/v) methanol for QI, 30% methanol for QII and 40% methanol for QIV. The absorbance of the eluent was measured at 275 nm.

Results and Discussion

Production of quinone reductases

A time course of reductase activity measured with QIV in N-limited cultures is shown in Fig. 2. The activity reached a maximum of 35 U ml⁻¹ after 72 h. At this point of cultivation the ligninolytic enzyme system of *P. chrysosporium* is activated by nitrogen starvation (Keyser *et al.*, 1978; Ulmer *et al.*, 1983).

Vanillic acid and veratryl alcohol were tested as inducers of quinone reductase activity since they have been shown to induce the ligninolytic system (Leisola *et al.*, 1984). When 1.5 mM-vanillic acid was added to N-limited cultures after 3 d, a 4.1- to 4.5-fold increase in activity was observed after 5.5 d depending on the substrate used in the enzyme assay. These results are in accordance with those of Buswell *et al.* (1979). In C-limited cultures a 26- to 42-fold increase in reductase activity was found after addition of vanillic acid. This is consistent with the suggestion of Ander *et al.* (1980) that quinone reductases are involved in the catabolism of vanillic acid. The greater increase of activity in cultures grown under C-limited conditions may have resulted from their ability to use vanillic acid as a carbon source. Veratryl alcohol was only a weak inducer of reductase activity in N-limited cultures, and actually decreased the activity when added to C-limited cultures at the beginning of cultivation (data not shown).

Several quinones have been identified as reaction

Table 1. Quinone reductase activity in crude extracts of N-limited and C-limited cultures of *P. chrysosporium* with various inducers and substrates

Quinones QI, QII or QIV were used as inducers (1 mM) and as substrates in the enzyme assay (250 μ M). See Methods for details. Quinone reductase activity is expressed as U ml⁻¹ and represents the mean of two experiments.

Inducer	N-limited culture			C-limited culture		
	Enzyme substrate:			Enzyme substrate:		
	QI	QII	QIV	QI	QII	QIV
None	19	28	28	3.2	7.5	6.0
QI	14	19	22	13	20	19
QII	105	167	176	7.7	11	11
QIV	12	18	21	6.6	8.6	7.1

products of the lignin model compound veratryl alcohol or its methyl ether when oxidized by lignin peroxidase (Schmidt *et al.*, 1989). Three of these quinones were also tested as inducers of reductase activity at a concentration of 1 mM. Their addition to the cultures after 3 d did not affect the growth of the fungus. All these quinones were able to increase quinone reductase activity in either C-limited or N-limited cultures or in both of them. The highest activities were obtained in N-limited cultures containing QII (Table 1). In C-limited cultures, adding QI resulted in higher activities than adding QII. QI and QIV stimulated quinone reductase activity only in C-limited cultures.

A concentrate of crude cell extract obtained from N-limited cultures was subjected to IEF (pH 4–8) to obtain information about the number of quinone reductases produced by the fungus. Activity staining of this gel with QI, QII or QIV as substrates revealed at least 10 quinone reductases with isoelectric points from 4.4 to 6.6 (Fig. 3). This finding contradicts the results of Schoemaker *et al.* (1989), who suggested that different quinone reductases specifically reduce QI, QII or QIV.

Purification

An intracellular quinone reductase from *P. chrysosporium* mycelium grown in N-limited conditions in the absence of an inducer was purified 163-fold. Its specific activity was 65880 U mg⁻¹ and the recovery of activity was 0.8% (Table 2). During the purification, the quinone reductase activities were measured with QI, QII and QIV. However, data are shown only for QIV, since the purification did not result in a separation of reductases specific for QI or QII.

The first purification step was a precipitation with 10% PEG. The resulting pellet contained no quinone reductase activity and was discarded. Thirty-seven

Table 2. Purification of quinone reductases from *P. chrysosporium*

The enzyme activities shown were determined with QIV as substrate. The purification procedure was performed three times with the exception of the anion-exchange chromatography, which was done only once. The values from one representative experiment are shown.

	Volume (ml)	Activity (U ml ⁻¹)	Protein concn (µg ml ⁻¹)	Sp. act. (U mg ⁻¹)	Yield (%)	Purification factor
Crude extract	720	224	557	403	100	
PEG precipitation	880	156	228	685	85	1.7
Blue Sepharose	280	88	32	2 532	15	6.3
Phenyl Superose	3	2 008	55	36 670	3.7	89
Mono Q	1	1 323	20	65 880	0.8	163

Fig. 3. IEF of the quinone reductases from *P. chrysosporium*. Active fractions were analysed on IEF gels pH 4–8 and the reductases were detected by activity staining with quinone QIV. A, quinone reductases in the crude extract of N-limited cultures; B, quinone reductases purified by chromatography with Blue Sepharose; C, quinone reductases in the precipitate obtained by addition of 1.7 M-(NH₄)₂SO₄ to the fraction purified by Blue Sepharose; D, quinone reductases purified by chromatography with Phenyl Superose.

percent of the activity in the supernatant was adsorbed to Blue Sepharose in a batch process. Quinone reductase activity was eluted as one peak after packing the gel into a column. The addition of 1.7 M-(NH₄)₂SO₄ to the eluate precipitated 55% of the quinone reductase activity present. The reductases in this pellet were different from those in the supernatant, as shown by IEF (Fig. 3) and were not purified further. The supernatant was applied to a Phenyl Superose column for hydrophobic interaction chromatography. The quinone reductases eluted from this column as a single peak at a salt concentration of 0.97 M. Final purification was performed by an anion-exchange chromatography. In this step, the quinone reductases eluted as a single peak at a salt concentration of 0.14 M.

Characterization

Isoelectric focusing of the quinone reductases purified by Phenyl Superose chromatography revealed four bands with quinone reductase activity (Fig. 3, lane D). Their

isoelectric points were 5.7, 5.9, 6.0 and 6.3.

SDS-PAGE and silver staining of quinone reductases further purified by anion-exchange chromatography revealed two bands, with molecular masses of 69 kDa and 29 kDa. To identify the strong band at 69 kDa as a quinone reductase, two-dimensional electrophoresis was performed. Reductases purified by Phenyl Superose chromatography were separated in the first dimension by IEF (pH 4–6.5). Subsequent SDS-PAGE in the second dimension indicated that all four bands visualized by activity staining after IEF had the same molecular mass of 69 kDa. To determine their molecular mass by gel filtration, the quinone reductases purified by Phenyl Superose chromatography were applied to a Superose 12 column. They eluted from this column as a peak corresponding to 47 kDa.

The difference between the molecular masses obtained by gel filtration and SDS-PAGE may be explained by the fact that the molecular mass determined by gel filtration depends on the shape of the molecule, whereas in SDS-PAGE the mobility of a protein depends on its charge. A

Table 3. Substrate specificity relative to QIV of quinone reductases from *P. chrysosporium* purified by hydrophobic interaction chromatography

Substrate	Relative rate of reduction (%)
QIV	100*
QI	49
QII	91
QIII	56
Methylbenzoquinone	34
3,5-Di- <i>t</i> -butyl-1,2-benzoquinone	92
2,3-Dimethoxy-5-methyl-1,4-benzoquinone	46
Tetramethyl-1,4-benzoquinone	0
2,6-Dimethyl-1,4-benzoquinone	7
1,4-Benzoquinone	80

*100% corresponds to 17 U ml⁻¹.

similar dual molecular mass effect has been reported in the case of a protopectinase (Sakai, 1988).

The quinone reductases were most active at pH 8.5 with QI as substrate, at pH 7.3 with QII and at pH 8.0 with QIV. At pH 5.0, activity with all three substrates was reduced to 25% of the maxima observed at the higher pH values. When stored for 1 d at 4 °C or frozen overnight at -20 °C, the crude cell extract lost 50% of its activity. Similar losses occurred when extract was stored in the presence of 20% glycerol. The half-life of the purified reductase was 120 h at 4 °C and a protein concentration of 40 µg l⁻¹. The enzyme was stable for one week at -20 °C in the presence of 0.5 mM-NADH.

The substrate specificity was tested with various substituted benzoquinones (Table 3). The K_m for QIV, which was the best substrate, was 115 µM. The K_m for NADH was 5 µM. The K_m for QIV was also determined in the resuspended pellet containing the reductase fraction obtained by precipitation with 1.7 M-(NH₄)₂SO₄ (Fig. 3, lane C). These reductases showed a K_m of 11 µM for QIV. In contrast to the purified enzyme, this reductase fraction used not only NADH but also NADPH as a cosubstrate.

None of several NADH-oxidoreductase inhibitors [1 mM-5,5-diphenylhydantoin, 1 mM-diethylbarbituric acid, 0.05 mM-5,5-dithiobis-(2-nitrobenzoic acid), 0.05 mM dicumarol, 0.05 mM-quercetin] decreased the activity of the purified quinone reductases by more than 20%. In contrast, complete inactivation was achieved by adding 1 mM-DDT. In crude cell extracts, 1 mM-DDT inhibited the reduction of QI, II and IV completely, whereas the reduction of QIII was inhibited by only 17%. Partial inactivation (42%) by DTT was also observed with the reductase fraction precipitated with 1.7 M-(NH₄)₂SO₄. *P. chrysosporium* therefore produces at least one reductase which is specific for QIII and which is not

inhibited by DTT. Similar results were reported by Schoemaker *et al.* (1989), who postulated that *P. chrysosporium* produces different intracellular quinone reductases specific for each of the quinone substrates. However, in this study we discovered ten reductases with low substrate specificity.

Product formation

The production of hydroquinones from QI, II and IV by purified quinone reductases was monitored by HPLC analysis. The hydroquinones were identified by comparing their retention times to those of authentic reference compounds. Each of the hydroquinones formed was found to react non-enzymically to further products. It is not known whether these compounds are also formed under physiological conditions. Analysis of the metabolites of QI, II and IV in the culture supernatants of *P. chrysosporium* is in progress and the results will be reported elsewhere.

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