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ORIGINAL PAPER

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Identification and heterologous expression of the cytochrome P450 oxidoreductase from the white-rot basidiomycete *Coriolus versicolor*

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Abstract A cDNA encoding cytochrome P450 oxidoreductase (CPR) from the lignin-degrading basidiomycete *Coriolus versicolor* was identified using RT-PCR. The full-length cDNA consisted of 2,484 nucleotides with a poly(A) tail, and contained an open reading frame. The G+C content of the cDNA isolated was 60%. A deduced protein contained 730 amino acid residues with a calculated molecular weight of 80.7 kDa. The conserved amino acid residues involved in functional domains such as FAD-, FMN-, and NADPH-binding domains, were all found in the deduced protein. A phylogenetic analysis demonstrated that *C. versicolor* CPR is significantly similar to CPR of the basidiomycete *Phanerochaete chrysosporium* and that they share the same major branch in the fungal cluster. A recombinant CPR protein was expressed using a pET/*Escherichia coli* system. The recombinant CPR protein migrated at 81 kDa on SDS polyacrylamide gel electrophoresis. It exhibited an NADPH-dependent cytochrome *c* reducing activity.

Introduction

Lignin is the most abundant renewable aromatic polymer and is known as one of the most recalcitrant biomaterials on Earth (Crawford 1981; Sarkanen and Ludwig 1971). Only white-rot basidiomycetes are known to be responsible for the complete mineralization of this polymer (Crawford 1981; Eriksson et al. 1990; Gold et al. 1989; Kirk and Farrell 1987; Tien 1987). Initially, basidiomycetes attack lignin polymers extracellularly, utilizing nonspecific oxidative enzymes such as lignin and manganese peroxidases (LiP/MnP) (Hammel and Moen 1991; Wariishi et al. 1991), resulting in the formation of a variety of aromatic fragments that are further metabolized intracellularly. Therefore, fungi seemed to possess a superior intracellular system to metabolize a number of aromatic skeletons. Involvement of cytochrome P450 (P450)-catalyzed hydroxylation reactions in the degradation of aromatic pollutants, including polycyclic aromatic hydrocarbons, diphenyl ether herbicides, and dibenzothiophenes, by white-rot basidiomycetes has been reported (Bezalel et al. 1996; Hiratsuka et al. 2001; Ichinose et al. 1999, 2001a, b; Sutherland et al. 1991). Since eukaryotic monooxygenase systems require not only P450s but also P450-reducing proteins (Oritz de Montellano 1995), biochemical information on P450-supporting and related systems is important in the understanding of the fungal ability to degrade a wide range of aromatic compounds.

NADPH-dependent cytochrome P450 oxidoreductase (CPR) is a membrane-bound protein containing 1 mol each of FAD and FMN (Iyanagi and Mason 1973; Kasper 1971; Phillips and Langdon 1962). It shuttles electrons from NADPH via its FAD and FMN prosthetic groups to P450. Thus, this class of enzyme is thought to be essential to activate monooxygenase systems. To date, the cloning of a basidiomycetous CPR gene has been reported only from *Phanerochaete chrysosporium* (Yadav and Loper 2000b).

In the present study, we report the isolation and characterization of a cDNA clone encoding CPR from the other white-rot basidiomycete, *Coriolus versicolor*, in which substrate-mediated induction of cytochrome P450 has been reported (Ichinose et al. 2001a). A recombinant CPR protein expressed using a pET/*Escherichia coli* system exhibited an NADPH-dependent cytochrome *c* reducing activity. This is the first report showing the expression of a basidiomycetous CPR gene in an enzymatically active form.

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Materials and methods

Organism and culture conditions

C. versicolor (IFO 30340) was grown from hyphae-inocula at 30°C in a stationary culture (10 ml medium) under air. The medium (pH 6.0) used in this study was previously described with 1% glucose (HC) and 1.2 mM (LN) or 12 mM (HN) ammonium tartrate as the carbon and nitrogen sources, respectively (Ichinose et al. 1999; Kirk et al. 1978). Thus, the fungus was incubated in either HCHN or HCLN medium.

cDNA synthesis and RT-PCR

After a 5-day incubation, total RNA was isolated from *C. versicolor* using an RNeasy Plant Mini Kit (QIAGEN). The total RNA was heat-treated at 70°C for 10 min and rapidly chilled on ice immediately before the RT reaction. The RT reaction (25 µl) was initiated by the addition of 200 U of Superscript II reverse transcriptase (Gibco-BRL) and 10 U of RNasin (Gibco-BRL) in 1× Superscript II reverse transcriptase buffer containing 10 mM dithiothreitol, 0.5 mM dNTPs, 0.5 µg of the total RNA and 20 pmol oligo$(dT)_{18}$ primer, followed by extensions at 45°C for 10 min and 50°C for 60 min. After the cDNA synthesis, the reaction mixture was diluted 4-fold with diethylpyrocarbonate-treated water. The subsequent amplification of cDNA fragments was performed with the primer combination of cprI and cprII (Table 1). The PCR mixture (100 µl) contained 3 µl of cDNA solution, 2.5 U of *TaKaRa Ex Taq* (TaKaRa), 1× *Ex Taq* buffer, 100 pmol each of cprI and cprII primers, and dNTPs at a final concentration of 200 µM. The PCR conditions were programmed as follows: 94°C for 3 min, 52°C for 2 min, and 72°C for 3 min for 1 cycle followed by 94°C for 20 s, 52°C for 1 min, and 72°C for 1.5 min for 20 cycles and with a final 5-min extension at 72°C. Nested PCR was performed with the primer combination of cprIII and cprIV (Table 1). The reaction mixture for nested PCR (20 µl) contained 0.5 µl of the initial PCR mixture, 0.5 U of *TaKaRa Ex Taq* (TaKaRa), 1× *Ex Taq* buffer, 20 pmol each of cprIII and cprIV, and dNTPs at a final concentration of 200 µM. The PCR conditions were programmed as follows: 94°C for 3 min, 52°C for 1 min, and 72°C for 1.5 min for 1 cycle followed by 94°C for 20 s, 52°C for 1 min, and 72°C for 1.5 min for 40 cycles and with a final 5-min extension at 72°C. All amplifications by PCR were performed using a DNA Thermal Cycler 2400 (Perkin-Elmer). PCR products were analyzed by electrophoresis on a 1.5% agarose gel and visualized using Molecular Imager FX (Bio-Rad).

Cloning and sequencing

After purification using 1.5% agarose gel and a QIAquick Gel Extraction Kit (Qiagen), each PCR product was cloned into the pGEM-T Easy vector (Promega), then transformed into *E. coli* strain JM109 competent cells. Positive clones were selected by blue-white screening. Plasmids were isolated from positive clones using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced with an automated DNA Sequencer (SQ5500; Hitachi) using a Thermo Sequenase Primer Cycle Sequencing Kit (Amersham Pharmacia). The nucleotide and deduced amino acid sequences were analyzed using the FASTA or BLAST search programs.

Determination of full-length cDNA

The nucleotide sequence of both the 5′- and 3′-regions was determined by the rapid amplification of cDNA ends (RACE) method (Frohman 1993). The full-length cDNA encoding CPR from *C. versicolor* was assigned DDBJ accession number AB065368.

Construction of expression vector and preparation of cell-free extracts

The expression vector was constructed using the pET-30 expression system. The coding region for CPR was amplified from plasmid DNA that contained cDNA encoding CPR including 5′- and 3′-non-coding regions in plasmid pUC18. The amplification of cDNA fragments was performed with the primer combination of *Nde*I-Freduc (5′-CCCATATGGCGATCGCCTCGTCCTCCT-3′) as the upstream primer and *Eco*RI-Rreduc (5′-CGAATTCGAGC-

Table 1 Amino acid sequences of conserved regions found in cytochrome P450 oxidoreductase (CPR) and nucleotide sequences of the primers designed for CPR identification from *Coriolus versicolor*. *I* Inosine, *Y* = C or T, *W* = A or T, *R* = A or G, *K* = G or T, *S* = C or G

Organism													Accession
	FMN-binding region-1												
Phanerochaete chrysosporium	23	F	Y	G	S	Q	T	G	T				AF193060
Aspergillus niger	69	F	Y	G	S	Q	T	G	T				S38427
Cunninghamella elegans	75	F	Y	G	S	Q	T	G	T				JC7192
Rhizopus stolonifer	45	F	Y	G	S	Q	T	G	T				AF290425
cpr I	5′-	TTY	TAY	GGI	WSI	CAR	ACI	GGI	AC-3′				
	FMN-binding region-2												
P. chrysosporium	78	T	Y	G	E	G	E	P					AF193060
A. niger	124	T	Y	G	E	G	E	P					S38427
C. elegans	130	T	Y	G	E	G	E	P					JC7192
R. stolonifer	100	T	Y	G	E	G	E	P					AF290425
cpr III	5′-	ACI	TAY	GGI	GAR	GGI	GAR	CC–3′					
	FAD-binding region-2												
P. chrysosporium	453	L	Q	P	R	Y	Y	S	I	S	S	S	AF193060
A. niger	446	L	Q	P	R	Y	Y	S	I	S	S	S	S38427
C. elegans	459	L	Q	P	R	Y	Y	S	I	S	S	S	JC7192
R. stolonifer	426	L	Q	P	R	Y	Y	S	I	S	S	S	AF290425
cpr II				3′-	KCI	ATR	ATR	WSI	TAI	W SI	WSI	WSI-5'	
cpr IV	3′-	RAI	GTY	GGI	KCI	ATR	ATR	WSI	TA-5′				

ACCTAGCTCCATACATCCG-3′) as the downstream primer. The PCR mixture (100 µl) contained 0.1 µg of plasmid DNA, 2.5 U of *Pyrobest* DNA polymerase (TaKaRa), 1× *Pyrobest* buffer II, 100 pmol each of *Nde*I-Freduc and *Eco*RI-Rreduc, and dNTPs with a final concentration of 200 µM. The PCR conditions were programmed as follows: 94°C for 3 min, 65°C for 1 min, and 72°C for 3 min for 1 cycle followed by 94°C for 20 s, 72°C for 3 min for 29 cycles and with a final 5-min extension at 72°C. The resultant cDNA fragments were digested by *Nde*I and *Eco*RI, and ligated into the pET-30 plasmid that had been treated with the same endonucleases. The recombinant plasmid obtained was then transferred into *E. coli* BL21 (DE3) pLysS (TaKaRa). The transformant was grown in LB broth containing kanamycin (50 µg/ml) and chloramphenicol (30 µg/ml) with shaking (200 rpm) at 37°C until an OD_{600} of 0.6. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was then added to the culture to a final concentration of 0.2 mM. After the addition of IPTG, bacterial cells were incubated with shaking (200 rpm) at 27°C. For the preparation of cell-free extracts, bacterial cells were collected by centrifugation at 10,000 rpm for 10 min at 4°C and washed with 50 mM potassium phosphate buffer (pH 7.4). Cells were resuspended in 1 ml of potassium phosphate buffer (pH 7.4) – standardizing cell density (OD_{600} =3.6) – and disrupted by sonication. After centrifugation at 15,000 rpm for 30 min, supernatants were used as cell-free extracts.

Fig. 1 Multiple alignment of the amino acid sequence of *Coriolus versicolor* cytochrome P450 oxidoreductase (CPR) with several known amino acid sequences of CPRs. *Cver C. versicolor*, *Pchr Phanerochaete chrysosporium*, *Cele Cunninghamella elegans*, *Rsto Rhizopus stolonifer*, *Anig Aspergillus niger*. The proposed functional domains are indicated above the sequence

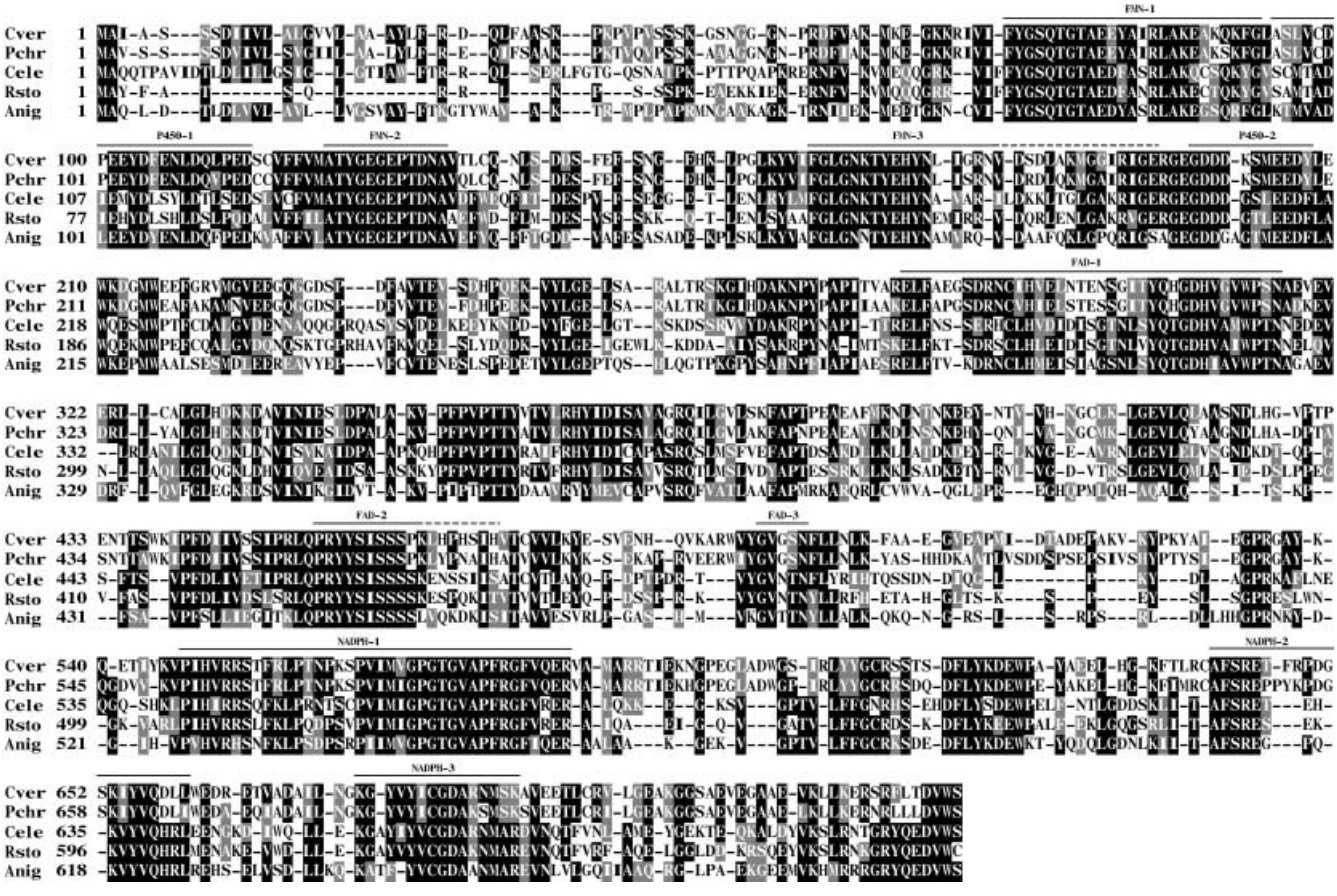
Assay for NADPH-cytochrome *c* reducing activity

NADPH-cytochrome *c* reductase activity was measured spectrophotometrically at room temperature. The reaction mixtures (1 ml) consisted of cytochrome *c* (10 µM), EDTA (0.5 mM), NADPH (30 µM), and 30 µl of cell-free extracts in 50 mM potassium phosphate (pH 7.4). The reaction was initiated by adding NADPH. The rate of cytochrome *c* reduction was calculated from the change in absorbance at 550 nm using an extinction coefficient for reduced minus oxidized cytochrome *c* of 21 $mM^{-1}cm^{-1}$ (Madyastha and Coscia 1979). The concentration of the ferric cytochrome *c* was determined using an ε_{410} of 106 $mM^{-1}cm^{-1}$ (Margoliashi and Frohwirt 1959).

Results

A cDNA encoding CPR from *C. versicolor*

For PCR amplifications of cDNA(s) encoding CPR from *C. versicolor*, inosine-containing degenerate primers were designed from the conserved regions shown in Table 1. Total RNA was isolated from *C. versicolor* grown at 30°C for 5 days under ligninolytic culture conditions. After the RT reaction, an initial PCR was performed with the primer combination cprI and cprII. Discrete DNA fragments from the initial PCR were undetectable on 1.5% agarose gel stained with ethidium bromide, showing only a smeared band. In order to detect specific cDNA fragment(s) encoding CPR, nested PCR was then performed with the primer combination cprIII and cprIV; one major cDNA band of approximately 1,000 bp in length was detected.



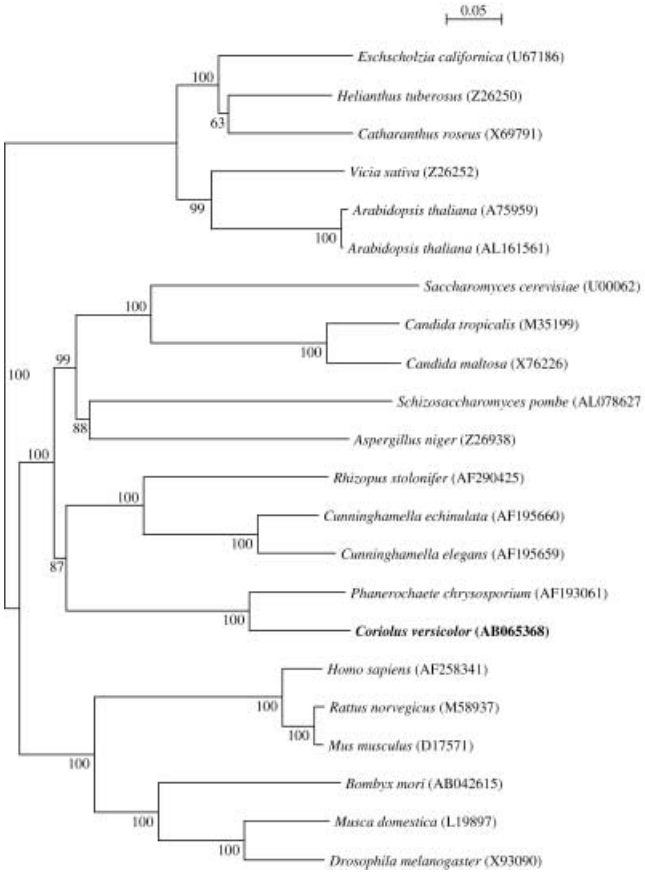


Fig. 2 Phylogenetic tree for cytochrome P450 oxidoreductases. Distance analysis and tree construction was attempted by the neighbor-joining method. Numbers represent bootstrap values for 100 replications. The *scale bar* indicates 5% change estimated sequence difference. Accession numbers are indicated in parentheses

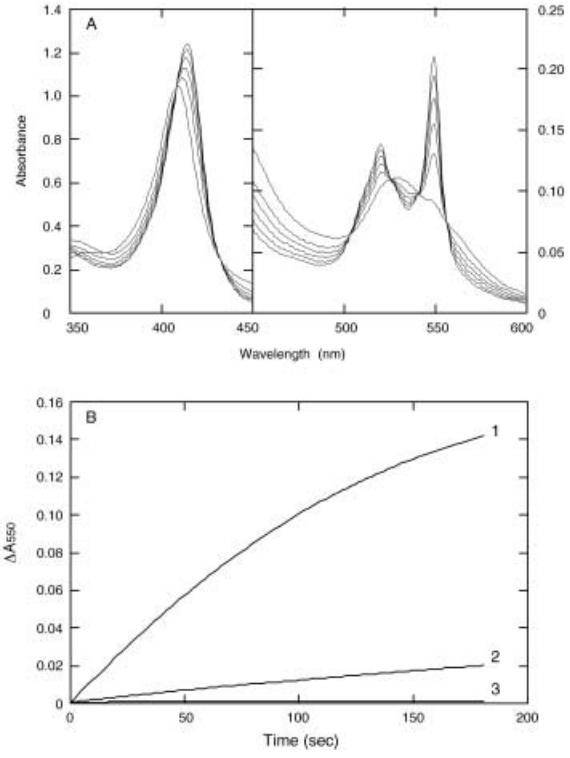


Fig. 3A, B Cytochrome *c* reduction by cell-free extracts. **A** Reaction mixture (1 ml) consisted of cytochrome *c* (10 μM), NADPH (30 μM), EDTA (0.5 mM), 30 μl of cell-free extract in 50 mM potassium phosphate (pH 7.4). Cell-free extracts were prepared from bacterial cells incubated for 12 h with isopropyl-1-thio-β-D-galactopyranoside (IPTG). The absorption spectrum was obtained at 30-s intervals after the addition of NADPH. **B** Reaction mixture (1 ml) consisted of cytochrome *c* (10 μM), NADPH (*1* 30 μM, *2* 30 μM, *3* 0 μM), EDTA (0.5 mM), 30 μl of cell-free extract in 50 mM potassium phosphate (pH 7.4). Cell-free extracts were prepared from bacterial cells incubated for 12 h with (*1* and *3*) or without (*2*) IPTG. The time course of cytochrome *c* reduction was monitored at 550 nm

Analysis of the DNA sequence of the cDNA fragment revealed that it consisted of 1,010 nucleotides. The deduced amino acid sequence from the cDNA obtained from the nested PCR showed high homology to a series of known CPR sequences. The nucleotide sequence of a full-length cDNA was then determined using 3′- and 5′-RACE methods. The full-length cDNA (*CvCPR*) consisted of 2,484 nucleotides with a poly(A) tail, and contained an open reading frame (ORF) that starts with an ATG codon and extends as far as the TAG stop codon at position 2,193 (DDBJ accession number AB065368). The ORF encodes a protein of 730 amino acids with a predicted molecular weight of 80.7 kDa. The amino acid sequence of CPR from *C. versicolor* was found to be enormously similar to CPR from *P. chrysosporium*, with 81% identity (Fig. 1). A phylogenetic comparison of several CPR protein sequences indicated that the CPRs from *C. versicolor* and *P. chrysosporium* are phylogenetically close (Fig. 2).

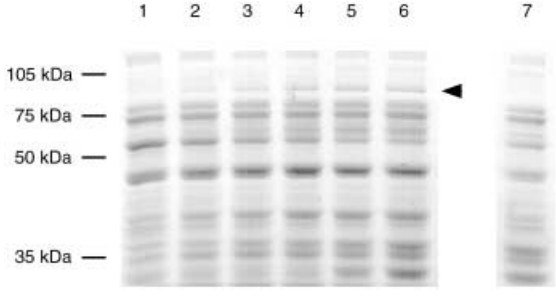


Fig. 4 Expression of recombinant CPR in *Escherichia coli*. *E. coli* cells were incubated with IPTG for 1 h (*lane 1*), 2 h (*lane 2*), 4 h (*lane 3*), 7 h (*lane 4*), 12 h (*lane 5*), 24 h (*lane 6*), or without IPTG for 12 h (*lane 7*) at 27°C. After incubation, cell-free extracts were analyzed by 10% SDS-PAGE and stained with Coomassie brilliant blue. The *arrowhead* indicates the position of recombinant CPR

Heterologous expression of *CvCPR*

The constructed expression vector containing the ORF encoding 730 amino acids was designed to express the recombinant protein without any additional tags. As shown in Fig. 3A, cytochrome *c* reduction was observed using cell-free extracts from CPR-expressing *E. coli* cells. SDS-PAGE analysis revealed that the recombinant

protein migrated at 81 kDa, consistent with the molecular weight predicted from the deduced protein (Fig. 4). Time-dependent production of CPR protein correlated well with time-dependent activity of the reduction of cytochrome *c* after IPTG addition. Cytochrome *c* reductase activity of 117 pmol min^{-1} μl^{-1} of cell-free extract 1 h after the addition of IPTG increased to 1,333 pmol min^{-1} μl^{-1} after a 24 h incubation. The initial cytochrome *c* reducing rate of CPR-expressing cells was 10-fold higher than that from non-expressing cells (Fig. 3B), strongly suggesting that the recombinant CPR was expressed in an enzymatically active form without any modification of the cDNA.

Discussion

Basidiomycetous cytochrome P450s have been suggested to be involved in the intracellular conversion of certain environmentally persistent aromatic compounds via hydroxylation reactions that cause a significant reduction in their ionization potential and an increase in their water-solubility (Bezalel et al. 1996; Hiratsuka et al. 2001; Ichinose et al. 1999, 2002b; Masaphy et al. 1996). Thus, these hydroxylated products are susceptible to further metabolic conversion. Although monooxygenase systems are thought to be indispensable for the superior ability of basidiomycetes to unilaterally metabolize a number of recalcitrant aromatic compounds, only limited information is available so far. Recently, P450 genes specifically expressed upon the addition of aromatic pollutants were partially cloned from the white-rot basidiomycete, *C. versicolor* (Ichinose et al. 2002a). Furthermore, it was very recently reported that at least 97 P450 genes exist in the other lignin-degrading basidiomycete, *P. chrysosporium* (via BLAST searches of the partial genome sequence of this fungus). This observation abolishes earlier notions that fungi were P450-poor and leads to new questions about the role and regulation of such numerous P450s in fungi (Nelson 2001).

Since CPR plays an important role in sustaining the catalytic cycle of P450s by catalyzing the electron transfer from NADPH to the P450 heme iron, we attempted to isolate and characterize a cDNA encoding CPR from *C. versicolor*. In addition, CPR has been reported to act as an electron donor for cytochrome b_5 (Enoch and Strittmatter 1979), heme oxygenase (Schacter et al. 1972), squalene epoxidase (Ono et al. 1977), and the fatty acid elongation system (Ilan et al. 1981). Furthermore, it has been suggested that CPR initiates lipid peroxidation by a one-electron reduction of molecular oxygen (Sevanian et al. 1990). Interestingly, lipid peroxidation was reported to be involved in the degradation of polycyclic aromatic hydrocarbons by basidiomycetes (Bogan and Lamar 1995; Moen and Hammel 1994). Because of its common and multiple roles as an electron donor, CPR is thought to be one of the most important enzymes in the fungal oxidation system.

Amplification of a cDNA encoding CPR from *C. versicolor*

CPR enzymes have been shown to contain several functionally important domains, such as FAD-, FMN-, and NADPH-binding domains. Thus, several conserved amino acid residues are found in functional domains of CPRs from a range of microorganisms (Porter and Kasper 1985, 1986; Smith et al. 1994; Sutter et al. 1990). Utilizing degenerate primers designed from these conserved regions, one major cDNA band of approximately 1,000 bp in length was detected, which was the size expected from the distance between FMN-2 and FAD-2 domains, as shown in several CPRs (van den Brink et al. 1995; Kargel et al. 1996; Miles 1992; Sutter and Loper 1989; Sutter et al. 1990; Yabusaki et al. 1988; Yadav and Loper 2000a, b; Yamano et al. 1989).

Sequencing and identification of a cDNA encoding CPR

The full-length cDNA (*CvCPR*) consisted of 2,484 nucleotides with a poly(A) tail, and contained an open reading frame (ORF) that starts with an ATG codon and extends as far as the TAG stop codon at position 2,193. The *CvCPR* has a G+C content of 60 %. A high G+C content is observed in many cDNAs isolated from basidiomycetes (Akileswaran et al. 1999; Ichinose et al. 2001a; Iimura and Tatsumi 1997; Raeder and Broda 1984). The ORF encodes a protein of 730 amino acids with a predicted molecular weight of 80.7 kDa. The amino acid sequence of CPR from *C. versicolor* was found to be enormously similar to CPR from *P. chrysosporium*, being 81% identical (Fig. 1). However, it was reported that other ligninolytic enzymes from *C. versicolor*, such as LiP and MnP, share only 50–60% identity with those of *P. chrysosporium* (Cullen 1997). It can be inferred from these results that fungal CPR and ligninolytic enzymes such as LiP and MnP, share a different evolutionary history. A phylogenetic comparison of several CPR protein sequences indicated that the CPRs from *C. versicolor* and *P. chrysosporium* are phylogenetically close, and share the same major branch in the fungal cluster (Fig. 2). In general, fungi are considered to be taxonomically close to plants. However, reports based on phylogenetic analysis of rRNA suggest that animal and fungal lineages share a more recent common ancestor than either does with the plant lineage (Wainright et al. 1993). Our comparison of CPRs also confirmed that basidiomycetes are phylogenetically closer to animals than to plants (Fig. 2).

Functional domains in the CPR from *C. versicolor*

It is believed that fusion between ancestral flavoprotein genes, the FMN-containing bacterial flavodoxin and the FAD-containing ferredoxin $NADP^+$ reductase genes, generated the CPR-encoding gene that has evolved in

higher organisms. This explains the high similarities found in FAD-, FMN-, and NADPH-binding regions and their vicinities among CPRs (Porter and Kasper 1985, 1986; Smith et al. 1994; Sutter et al. 1990). Although the overall sequence similarity of *C. versicolor* CPR with CPRs from several microorganisms other than *P. chrysosporium* was less than 50%, e.g., *Cunninghamella elegans* (44% identity), *Rhizopus stolonifer* (45%), and *Aspergillus niger* (44%), the amino acid sequences encoding the functional domains, such as FMN-binding domains (FMN-1, -2, and -3), FAD-binding domains (FAD-1, -2, and -3), and NADPH-binding domains (NADPH-1 and -2), exhibit a very high homology (Fig. 1) (Porter and Kasper 1985, 1986; Sem and Kasper 1993).

From the alignment analysis, the presence of P450-1 and -2 regions in *C. versicolor* CPR was also proposed. The P450-1 region was located between FMN-1 and -2 and the P450-2 region was in the vicinity of FMN-3. It has been proposed that the acidic amino acid residues found in the vicinity of the FMN binding regions play an important role in the interaction of CPR with cytochrome P450 and cytochrome *c* (Shen and Kasper 1995). A series of acidic amino acid residues (Asp99, 104, 109, 198–200, and 206; Glu101, 102, 106, 113, 204, and 205) are found in either P450-1 or -2 (Fig. 1). Furthermore, four invariantly conserved aspartic acid residues were also identified in *C. versicolor* CPR; Asp99 in P450-1 and Asp198, 199, and 206 in P450-2. These data strongly suggest that *CvCPR* encodes functionally active cytochrome P450 oxidoreductase.

Heterologous expression of *CvCPR*

The expression of enzymatically active *C. versicolor* CPR was successfully achieved using a pET/*E. coli* system (Figs. 3 and 4). The cytochrome *c* reduction cell-free extract from *CvCPR*-expressing *E. coli* was dependent on NADPH but not NADH (data not shown). This result was consistent with the prediction from amino acid sequence analysis showing the existence of an NADPH-binding domain (Fig. 1). All these results suggest that *C. versicolor* CPR is capable of transferring an electron from NADPH to P450 of basidiomycetes.

Fungal monooxygenase system

Recently, we showed that *C. versicolor* has a series of P450 genes. Some of them were found to be up-regulated by exogenous substrates (Ichinose et al. 2001a), suggesting that a chemical stress responsive system is involved in xenobiotic metabolism in *C. versicolor*. On the other hand, the mRNA level of CPR was almost unchanged in either chemically stressed or unstressed cells and under either primary or secondary metabolic conditions (data not shown). These results may indicate that CPR is constitutively produced and that it plays an important role as an electron donor for a series of P450 enzymes in *C. versicolor*. However, it was recently reported that substrate-mediated co-induction of P450 and CPR occurs in other fungi (van den Brink et al. 2000). Identification of other possible CPR genes and enzymes from *C. versicolor*, especially those involved in stress-response, is now being attempted as well as investigation into the interaction between CPR and P450s from *C. versicolor*.

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