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P450 Redox Enzymes in the White Rot Fungus *Phanerochaete chrysosporium*: Gene Transcription, Heterologous Expression, and Activity Analysis on the Purified Proteins

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Abstract

With an aim to understand the cytochrome P450 enzyme system in the white rot fungus *Phanerochaete chrysosporium*, here we report molecular characterization of its P450 redox proteins including the primary P450 oxidoreductase (POR) and two alternate P450 redox proteins cytochrome b5 (cyt b5) and cytochrome b5 reductase (cyt b5r) in terms of transcriptional regulation and heterologous expression. The transcript abundance followed the order *POR* > *cyt b5r* > *cyt b5*. Interestingly, the three genes showed an overall higher expression in the defined carbon-limited cultures with low nitrogen (LN) or high nitrogen (HN) versus the carbon-rich malt extract (ME) cultures. cDNA cloning and analysis revealed the following deduced protein characteristics: cyt b5 (238 amino acids, 25.38 kDa) and cyt b5r (321 amino acids, 35.52 kDa). Phylogenetic analysis revealed that the cloned cyt b5 belongs to a novel class of fungal cyt b5-like proteins. The two proteins cyt b5 and cyt b5r were heterologously expressed in *E. coli* and purified using affinity-based purification in an active form. The POR was heterologously expressed in *Saccharomyces cerevisiae* and was also purified in active form as evidenced by its cytochrome c reduction activity. This is the first report on cloning, heterologous expression, and purification of the alternate redox proteins cyt b5 and cyt b5r in *E. coli* and on yeast expression of POR from this model white rot fungus.

Introduction

Phanerochaete chrysosporium has been the most intensively studied model organism for understanding the physiology, biochemistry, and genetics of biodegradation of lignin and

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toxic chemical pollutants by basidiomycetous white rot fungi. Peroxidases and oxygenases including P450 monooxygenases have been shown to play important roles as initial oxidizing systems in this white rot fungus for biotransformation of different xenobiotics [1, 2]. Whole genome sequencing has revealed that this fungus carries a large P450 contingent comprising of about 150 P450 genes, arranged in 16 gene clusters that are grouped under existing 12 cytochrome P450 (CYP) families and 11 fungal CYP clans [3, 4], and a single P450 reductase component.

The NADPH-dependent cytochrome P450 oxidoreductase (POR, EC 1.6.2.4), formerly abbreviated as CPR, is known to serve as a common electron donor to multiple monooxygenases in a typical microsomal P450 system, although multiple PORs have been reported in certain plants and even fungi [5, 6]. The electron transfer proceeds from NADPH to the P450 heme via FAD and FMN domains of the POR. Typical type II microsomal eukaryotic P450 mono-oxygenases primarily obtain both the electrons, needed for their monooxygenation reaction, from the POR, although involvement of an alternate electron transfer mechanism via the cyt b5 reductase-cyt b5 chain in providing one of the two electrons (the second electron) from NADH to the P450 monooxygenase has been known [7]. Cytochrome b5 reductase (EC 1.6.2.2, cyt b5r), a membrane-bound flavoprotein containing a single FAD as a prosthetic group, catalyzes the reduction of cytochrome b5 (cyt b5) utilizing NADH as an electron donor. Cytochrome b5 (cyt b5) is known to be involved in a number of oxidative reactions, which include metabolism of fatty acids, steroids, and endogenous compounds. The role of cyt b5 as an obligate partner and modifier in xenobiotic biotransformation has been documented for higher eukaryotes [7], but such information is not available for filamentous fungi. Whereas *cyt b5* and *cyt b5r* are located on genome scaffold 4 (whole genome version 2.0), the *POR* gene is located on scaffold 12 (whole genome version 2.0) of the *P. chrysosporium* genome. None of the three genes co-localize with any of the known 16 P450 clusters in the *P. chrysosporium* genome [3, 4].

In addition to POR and the cyt b5r-cyt b5 complex, cytochrome P450 enzymes can also receive electrons from other protein partners in eukaryotes depending on their intracellular location and their physiological function. For example, type I mitochondrial P450 systems obtain electrons from adrenodoxin reductases (AdR). Specifically, AdRs receive electrons from NADPH and transfer them to the P450 enzymes via the [2Fe-2S]-ferredoxin-type carrier adrenodoxin [8]. Type III P450s are self sufficient and do not require molecular oxygen or external electron source. Type IV proteins such as P450nor (*nicA*) can transfer electrons directly from NADH/NADPH to its substrate nitric oxide [9]. Type I, III, and IV P450 proteins have not been identified in the P450ome of *P. chrysosporium*.

Considering the extraordinarily large nature of the P450 monooxygenase contingent (~150 P450 genes) in *P. chrysosporium*, the single P450 oxidoreductase (POR) enzyme present in this organism could be insufficient to cater to the electron transfer needs of all the P450 mono-oxygenases. Presently, little or no information is available with respect to gene regulation and function of the P450 redox proteins of the P450 enzyme system in *P. chrysosporium*. Therefore, characterization of these enzymes, including the primary redox protein POR and the alternate redox proteins cyt b5 and cyt b5 reductase, will facilitate understanding of the redox mechanisms during P450 monooxygenation reactions in this model biological system.

Materials and Methods

Fungal Strain and Culture Conditions

Phanerochaete chrysosporium strain BKM-F-1767 (ATCC 24725) was routinely maintained on malt extract agar (Difco Laboratories, USA).

Analysis of Gene Transcription

Gene transcripts for *POR*, *cyt b5*, and *cyt b5r* were quantified by real-time quantitative RT–PCR (qRT–PCR) using gene-specific primers (supplemental table) as described previously [10, 11]. Glyceraldehyde-3-phosphate dehydrogenase (*GPD*) was used as the internal reference gene for transcript quantification. Time course of transcription was compared for the mycelia grown in defined low nitrogen (LN) medium (2.4 mM N, 1% glucose), defined high nitrogen (HN) medium (24 mM N, 1% glucose), and the complex malt extract (ME) medium (8 mM N, 2% glucose).

Whole Cell Protein Extracts and Microsomes from *P. chrysosporium*

Fungal cultures grown in LN, HN, and ME media were harvested on day 4 of incubation and snap frozen at −80°C. The frozen mycelium was macerated in liquid nitrogen and vortexed with glass beads (seven pulses of 30 s each). The extract was clarified twice by centrifugation at 11,000×*g*, to obtain whole cell protein extract. The extract was subjected to ultracentrifugation (100,000×*g*) for 1 h to isolate microsomes, using a published procedure [12].

cDNA Cloning for *cyt b5* and *cyt b5r*

Full-length cDNAs of *cyt b5* and *cyt b5r* were isolated by RT–PCR using gene-specific primers (supplemental table). Briefly, the total RNA (1.5 μg) isolated from day 4 ME culture was reverse transcribed using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen Corp., USA), followed by PCR amplification using *Pfu* ultra DNA polymerase (Stratagene, USA) as described previously [13].

DNA Sequencing and Bioinformatic Analysis of DNA Sequence Data

cDNA sequencing was performed as described previously [14]. The nucleotide sequences and deduced amino acid sequences were aligned using the CLUSTALW program at EMBL-EBI website (http://www.ebi.ac.uk/clustalw/) followed by editing and shading of the alignment using GeneDoc 2.0.1 Multiple Sequence and Alignment Editor software. Minimal evolution trees were constructed using the MEGA 2.1 software (http://www.megasoftware.net/) as described previously [3]. A bootstrap value of 1000 was set for tree construction. The cDNA sequences cloned in this study have been submitted to GenBank under the accession numbers AY862990 and AY835609.

Heterologous Expression, Purification, and Activity Analysis of the White Rot Fungus POR in *Saccharomyces cerevisiae*

P. chrysosporium POR cDNA earlier isolated in our laboratory [14] was cloned into the yeast expression vector pYES2.1/V5-His-TOPO (Invitrogen Corp., USA), in frame with the C-terminal histidine (His) tag sequence. The expression construct, confirmed for the sequence accuracy and correct orientation of the cloned *POR* cDNA, was transformed into *S. cerevisiae* Y300 (*MATa ade2-1 trp1-1 ura3-1 leu2-3, 112 his 3-11, 15 can1-100*) strain for protein expression. The expressed protein was affinity purified using Nickel–Nitriloacetic acid column (Ni–NTA) and the protein was detected by Western Blot analysis. A detailed version of the protein expression protocol has been provided in supplemental file 1. Functional activity of the recombinant fungal POR was determined following an established protocol [15]. Briefly, activity of the POR was determined based on its ability to reduce cytochrome c using NADPH as the electron donor by monitoring the increase in absorbance at 550 nm. The extinction coefficient used for the calculation was 21 mM^{-1} cm^{-1}.

Heterologous Expression, Purification, and Activity Analysis of the Fungal cyt b5 and cyt b5r in *E. coli*

Expression of the *P. chrysosporium* redox proteins, cyt b5 and cyt b5r, in *E. coli* was performed using pET30a(+) expression system (EMD Biosciences, Inc., USA). Both *cyt b5* and *cyt b5r* cDNAs were amplified using gene-specific primers (see Table 1 in supplemental information). After DNA sequencing to ensure correct nucleotide sequence, the cDNAs were directionally cloned into the pET30a(+) expression vector at the *Eco*RI and *Hind*III sites. The cloning strategy was such that the *cyt b5* construct expressed cyt b5 protein along with the in-frame N-terminal His tag, where as the *cyt b5r* construct expressed cyt b5r protein along with the in-frame N- and C- terminal His tags. The constructs were confirmed by performing restriction digestion and transformed into the *E. coli* BL21 derivative strain RosettaBlue DE3 (EMD Biosciences, Inc., USA). A common protocol was followed for expression of the two proteins unless specified otherwise [15]. Details on the protein expression and purification protocol are provided in supplemental file 1. Cytochrome b5 expression was confirmed by Western Blot analysis and based on determination of its typical oxidized and reduced absorption spectra. Functional activity of the cyt b5r was determined based on its ability to reduce cyt b5 using NADH as the electron donor, following an established protocol [15, 16].

Results and Discussion

Nutritional and Temporal Regulation of Transcription of the P450 Redox Proteins

The P450 electron transfer proteins in *P. chrysosporium* showed an overall difference in their relative transcript abundance (*POR* > *cyt b5r* > *cyt b5*) across the media conditions, but no tight temporal or nutritional regulation as these were expressed during both phases of growth (primary and secondary) in nutrient-limited conditions and the corresponding growth stages in nutrient-rich media conditions. In LN and HN cultures, the *POR* transcript number peaked on day 3 (HN) and day 4 (LN) (Fig. 1a). The ME cultures showed an overall lower expression as compared to LN and HN cultures. The level of expression in ME decreased over the first 5 days followed by a transient increase on day 6. However, based on Western blot analysis of the microsomes isolated from day 4 LN, HN, and ME cultures, the POR protein was detectable only under HN conditions, despite comparable transcript abundance in LN and HN conditions (see Fig. 2). This difference between the two culture conditions could likely be due to the differences in terms of translation efficiency or stability of the protein product.

Cytochrome b5 (*cyt b5*) showed similar transcriptional expression pattern as *POR*, with peaking on day 3 in HN cultures and day 4 in LN cultures. However, its expression differed from that of *POR* in HN cultures in that a second surge was observed from day 6. Expression in ME cultures remained low and was nearly constant through the different stages of growth (Fig. 1b).

Cytochrome b5 reductase (*cyt b5r*) expression in LN cultures showed a biphasic peaking, with the first peak coinciding with the transition to secondary metabolism (day 3 or 4) as in case of *POR* and *cyt b5* and the second peak in the late secondary metabolic phase (day 6). A biphasic peaking was also observed under HN growth conditions. However, the first peak appeared earlier (day 3) in HN cultures as compared to LN cultures (day 4). Expression in ME cultures remained almost unchanged with a slight peak on day 5 (Fig. 1c).

Activity of the P450 enzymes is known to depend on the kinetic efficiency as well as expression level of the electron transfer (redox) proteins that provide reducing equivalents in order to complete their catalytic cycle [17]. For instance, the activity of P450 benzoate para hydroxylase in *Aspergillus niger* has been shown to be enhanced by increasing the copy

number of its *POR* [18]. On the other hand, deletion of *POR* led to an increased sensitivity to benzoate in *Gibberella fujikuroi* [19]. Furthermore, it has been suggested that regulated expression of reductases selectively controls the activity of P450 monooxygenases in *Streptomyces coelicolor* [20]. It is therefore likely that the activity of P450 proteins at the functional level could be modulated, at least in part, via transcriptional regulation of the redox proteins in *P. chrysosporium*. In this context, a comparison of the transcriptional pattern of the three redox proteins observed in this study with our earlier observations on P450 monooxygenase genes (particularly CYP63 family *pc*-1, *pc*-2, and *pc*-3) in this organism [10, 13, 21, 22] under the same test nutrient conditions (LN, HN, ME), implies a co-ordinated expression of the two sets of proteins (the P450 redox proteins and the P450 monooxygenases). Particularly striking among the similarities are (i) peaking of transcript levels of CYP63 genes *pc*-1 and *pc*-2 on day 4 in LN conditions, (ii) an overall higher transcript levels (over the 8-day period) of all three CYP63 family member genes between day 2 and day 4 in HN conditions, and (iii) higher levels of *pc*-1 and *POR* expression on day 6 in ME conditions. This observation supports the working hypothesis that both the POR and the cyt b5/cyt b5r redox chain are involved, at least in part, in transferring electrons to the CYP63 family of P450 monooxygenase enzymes, in addition to transferring electrons to other uncharacterized P450 enzymes in this fungus. Nevertheless, the involvement of other uncharacterized oxidoreductases playing a role as redox partners to P450 monooxygenases in this fungus cannot be ruled out. Although, transcript abundance of the P450 system components (the monooxygenases and the redox proteins) varies with the nutrient levels, this is far from being a tight nutrient regulation of expression, a phenomenon reported as a hallmark for peroxidases in this organism [23]. Nevertheless, an overall reduced level of transcriptional expression of the P450 redox proteins in nutrient-rich ME medium (high organic nitrogen, high carbon) versus the nitrogen-rich HN medium (high inorganic nitrogen, low carbon) suggested a possible carbon-mediated regulation and/or regulation by nitrogen type (organic versus inorganic), albeit to a limited extent, for the three P450 redox proteins.

Structural, Phylogenetic, and Functional Aspects of the Redox Proteins cyt b5 and cyt b5r

The isolated cDNAs of *cyt b5* (717 bp) and *cyt b5r* (966 bp) encode 238 amino acid (aa) and 321 aa long deduced proteins, respectively. Other key structural features of the gene sequences are summarized in Table 1.

Alignment of the deduced amino acid sequence of the cloned cyt b5 with cyt b5 protein sequences from other organisms (including experimentally characterized and deduced proteins) and two additional putative cyt b5 (“cyt b5-like”) sequences predicted by the Joint Genome Institute (JGI) from the *P. chrysosporium* genome (whole genome version 1) revealed the presence of conserved residues particularly the HPGG (histidine, proline, glycine, glycine) motif in the heme-binding region that corresponded to amino acids H194 to G197 in the cloned protein (Fig. 3a). The deduced protein of the cloned cyt b5 gene of *P. chrysosporium* was found to be divergent from the other characterized eukaryotic cyt b5 proteins. First, this protein (238 aa) was longer by nearly 90 aa particularly on the N-terminus as compared to the cyt b5 proteins from other sources. As a result of this sequence extension, the HPGG motif that is usually located near the N-terminus (centering around 40–75 aa) was found downstream toward the C-terminus (centering around 190–210 aa) of the cloned cyt b5. Second, the TMpred analysis did not yield any transmembrane domain with significant score (the predicted weak region around 218 and 236 aa positions had a score of 378) in contrast to a transmembrane domain found in the other cyt b5 proteins. Interestingly, the BLAST search on other fungal genomes showed the presence of similar proteins (*E* value ≥ 9e-28) in other fungi with uncharacterized function. The first 50–60 aa residues in the cloned *P. chrysosporium* cyt b5 have no match in any of these longer cyt b5-

like proteins in other fungi suggesting a hitherto uncharacterized function for this N-terminal stretch of the protein. The phylogenetic tree clustering agreed with that based on the multiple alignments and showed three distinct clusters (Fig. 3c). The conventional cyt b5 and the hypothetical proteins from other organisms formed two separate groups and the two JGI-predicted protein models of *P. chrysosporium* formed the third group with a high bootstrap value of 99.

The *P. chrysosporium* cyt b5r protein showed the characteristic flavin-binding domain RXY(T/S)XX(S/N) that corresponded to the amino acid stretch R154 to S160 in the expressed mature protein (Fig. 3b). The isolated cyt b5r protein showed high sequence similarity with other cyt b5r proteins suggesting that they might possess common functional characteristics. Phylogenetic tree constructed using other known cyt b5r proteins suggested that the isolated *P. chrysosporium* cyt b5r protein was closest to the zygomycetous fungus *Mortierella alpina* homolog with a bootstrap value of 61 followed by the ascomycete *Schizosaccharomyces pombe* homolog (Fig. 3d).

Cytochrome b5 is usually reduced by the NADH-dependent cyt b5r. In addition to its involvement in lipid, plasmalogen, and sterol biosynthesis, cyt b5 can also provide the second electron required for the P450-dependent monooxygenation reactions [24, 25]. In this context, Sutter and Loper [26] showed that disruption of P450 reductase in yeasts did not prove to be lethal indicating that alternate proteins such as cyt b5 can mediate transfer of electrons to support CYP51 activity, as was later confirmed in vitro [27]. With the large contingent (~150 P450 genes) of simultaneously expressing P450 genes in *P. chrysosporium*, a role of alternate source(s) of electrons such as cyt b5 is highly likely in order to maintain the functionality of cytochrome P450s under different nutritional and biodegradation conditions. Considering that both *POR* and *cyt b5/cyt b5r* are constitutively expressed during the different phases of growth, the two redox enzyme systems may have a collective role to play in the overall electron transfer reactions in the P450-mediated physiological and biodegradation activities of this fungus. Relative role of these proteins could be resolved in future efforts, once the gene manipulation in this fungus is optimized for routine applications.

Heterologous Expression, Purification, and Activity Analysis of the P450 redox Proteins of *P. chrysosporium*

Expression of the POR protein in the yeast transformant gradually increased after induction with 2% galactose during the 8–16 h post-induction period (Fig. 4A1). Hence, subsequent purification of the expressed POR was carried out using 16 h-induced yeast cultures. The His-tagged recombinant POR protein (calculated molecular weight of 81.7 kDa) migrated as a single band on the SDS-PAGE gel, appearing between 66 and 97 kDa size protein standards in Western blot analysis (Fig. 4A1). Purification of the expressed POR using Ni–NTA agarose (Qiagen Inc., USA) column showed that the protein selectively elutes at 80 mM imidazole concentration in two different fractions (Fig. 4A2). Based on Western blot analysis the POR eluate fractions were visually free from any background protein bands (Fig. 4A3). The purified POR (7.4-fold purification) was found to be functionally active, as determined by cytochrome *c* reduction (cyt *c*) activity (specific activity of 91.7 U/mg; Table 2).

Both cyt b5 and cyt b5r were heterologously expressed in *E. coli.* Cytochrome b5 expressing cells appeared pinkish in color, an appearance typical of a functionally active cyt b5 protein. The recombinant cyt b5 protein from the cell free extract showed an absorption maximum at 413 nm (data not shown), which is characteristic of native functional form of this protein. Cell free extracts prepared from *E. coli* culture expressing cyt b5r showed reduction of cyt b5 with a specific activity of 1.84 U/mg (Table 2). Ni–NTA column-based purification

showed that the cyt b5 protein eluted in the third (E3) and fourth (E4) fractions (Fig. 4B1), corresponding to 80 and 150 mM imidazole concentrations, respectively, where as the cyt b5r protein eluted in the second (E2), third (E3), and fourth (E4) fractions (Fig. 4C1), corresponding to 80 mM imidazole concentration. The proteins separated at the expected molecular weights in the SDS-PAGE gel, 31 kDa for cyt b5 (Fig. 4B1) and 43 kDa for cyt b5r (Fig. 4C1). The eluate fractions of the recombinant cyt b5 and cyt b5r proteins were visually free from any contaminating background proteins based on Western blot analysis using anti-His antibodies (Fig. 4B2, C2). The purified cyt b5 protein showed the characteristic difference absorption spectrum with a peak at 412 nm for the oxidized spectrum and a peak at 424 nm when reduced with sodium dithionite (Fig. 5), suggesting that cyt b5 purification yielded a functionally active protein. The cyt b5r protein was purified (~690-fold) and the purified form was shown to be a functional protein based on its ability to reduce cyt b5 (Table 2). In vitro reduction of cyt b5 by cyt b5r, using NADH as the electron donor, suggested that these two proteins have the potential to form an active complex to perform the electron transferring role in this white rot fungus, as in other eukaryotes. Further, based on transcriptional expression of the two genes *cyt b5* and *cyt b5r* along with the functional ability of their encoded gene products to act as redox partners, it could safely be predicted that these two proteins could also be involved in transferring electrons to P450 enzymes in *P. chrysosporium* as in other higher eukaryotes [7]; a direct evidence for this mechanism is yet to be established in this organism.

In conclusion, this is the first report on heterologous expression of *P. chrysosporium* POR in a eukaryotic (yeast) host *S. cerevisiae* and native and heterologous expression of functionally active alternate P450 redox partners (cyt b5 and cyt b5r proteins) from a white rot fungus. Yeast *S. cerevisiae* expressing a functional white rot fungal POR could serve as an initial tool for developing whole cell yeast biocatalysts for P450 catalysis applications as well as in similar applications requiring reconstituted P450 enzyme system. Availability of the expressed P450 redox proteins would allow functional studies on the hitherto uncharacterized catabolic P450 monooxygenases of this model white rot fungus. In-depth understanding of transcriptional regulation of these redox proteins would further help in developing the white rot fungus as a more efficient biocatalyst for intended environmental and industrial biotechnological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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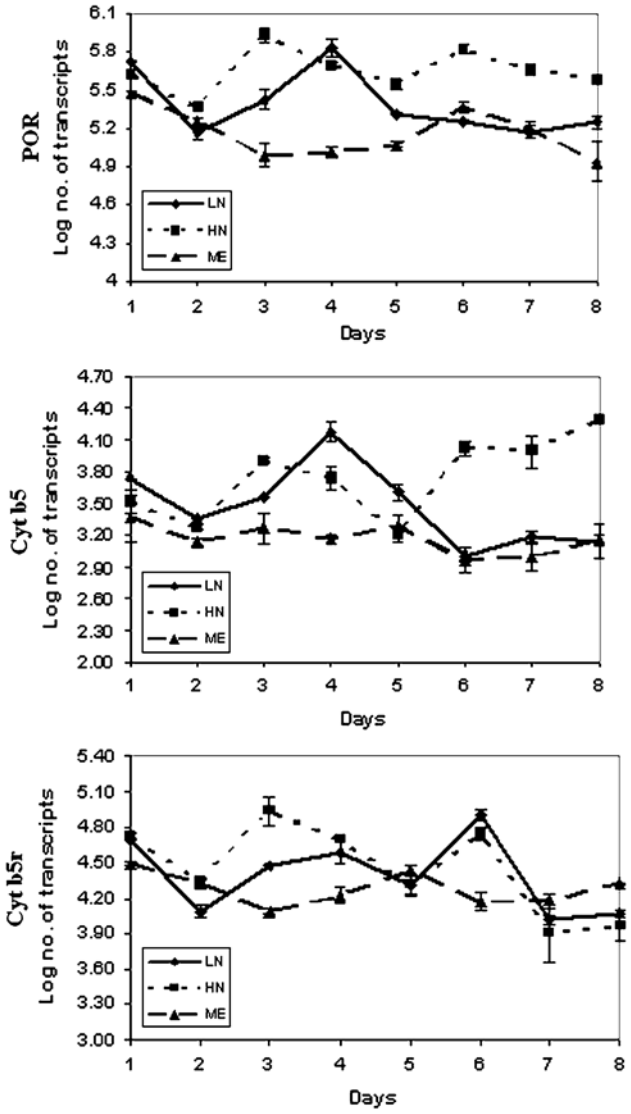

Fig. 1.

Time course of transcriptional expression of *POR*, *cyt b5*, and *cyt b5r* in *P. chrysosporium* under different nutrient media conditions: Total RNA extracted from fungal mycelia harvested from day 1 through day 8 cultures was subjected to real-time quantitative RT–PCR using gene-specific primers. Transcript numbers were calculated from a standard curve, prepared as described previously [10, 11]. Average log value of the transcripts was plotted against time. Abbreviations: LN = defined low nitrogen medium, HN = defined high nitrogen medium, ME = malt extract medium

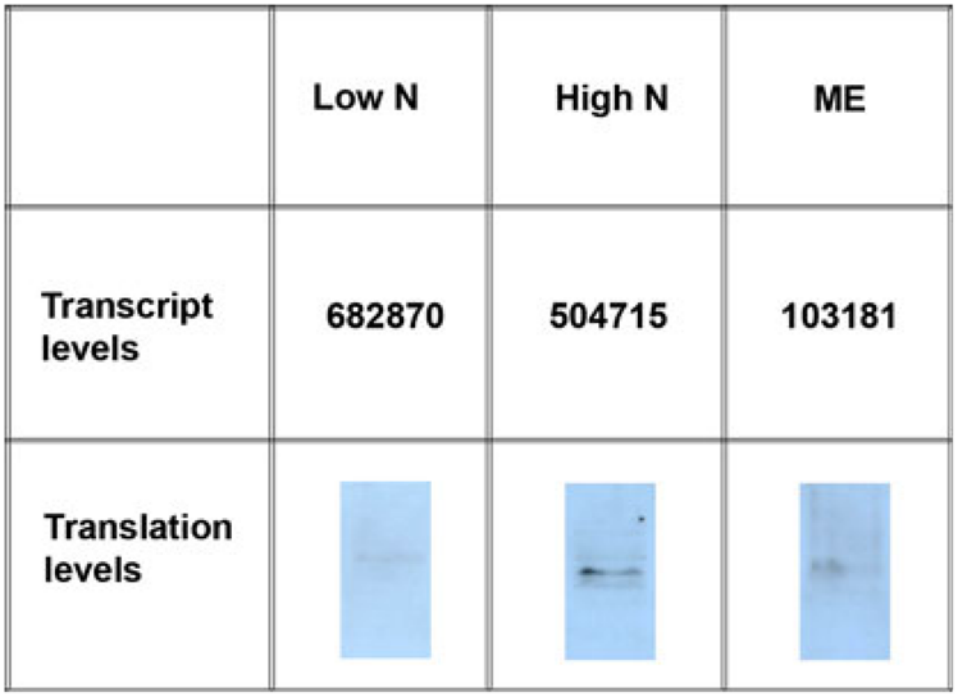

Fig. 2.

Transcript and protein levels of POR expressed in *P. chrysosporium* under different nutrient conditions. The transcript numbers were determined based on the real-time quantitative RT–PCR analysis under LN, HN, and ME conditions, as specified in Fig. 1 legend. The values obtained (in duplicates) were normalized against those of the control gene (*GPD*). Translational expression levels were analyzed by Western blot analysis. This involved separation of the corresponding microsomal protein extracts (50 μg protein) on a 10% SDS-PAGE gel followed by incubation with the crossreactive yeast anti-POR antibody and detection by autoradiography

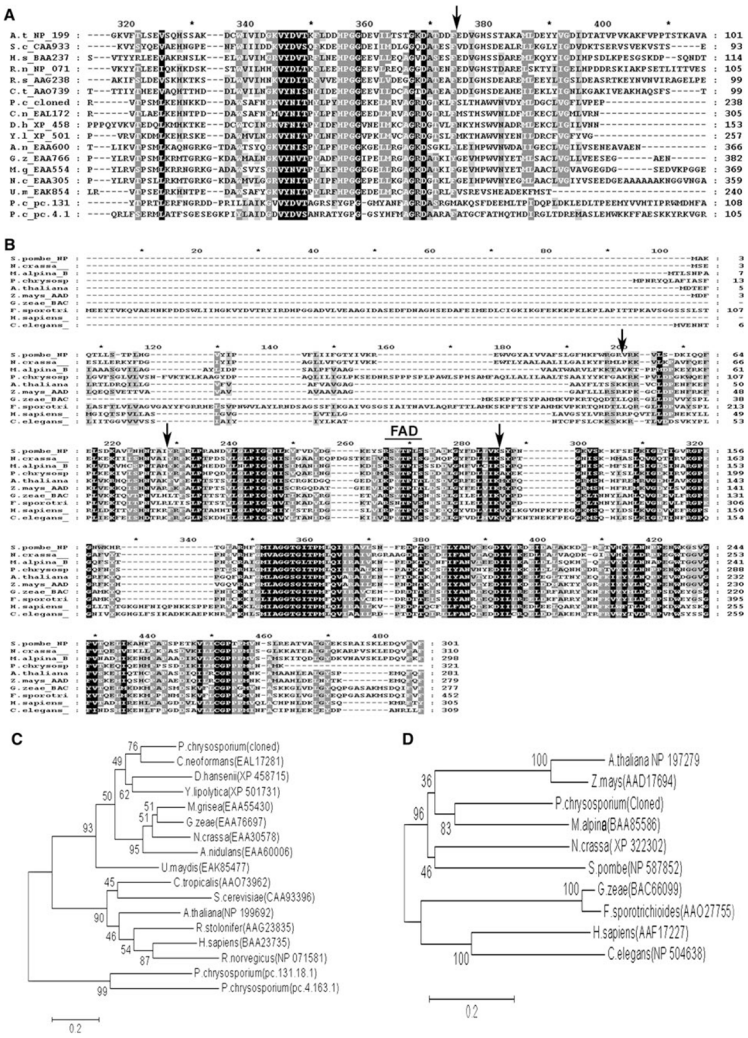

Fig. 3.

Amino acid (aa) sequence alignment and phylogenetic analysis of the cloned cyt b5 and cyt b5r proteins of *P. chrysosporium* against their homologs from other organisms. **a** Alignment of the HPGG motif context sequence of cyt b5 with corresponding aa sequence of the homologs from other organisms. **b** Amino acid sequence alignment of the isolated full-length cyt b5r with those from other organisms. **c** Minimum evolution tree of the cyt b5 proteins. d Minimum evolution tree of the cyt b5r proteins. Conserved residues are marked with a line over the aa sequence. *Arrows* indicate the location of introns in the corresponding genomic DNA sequence

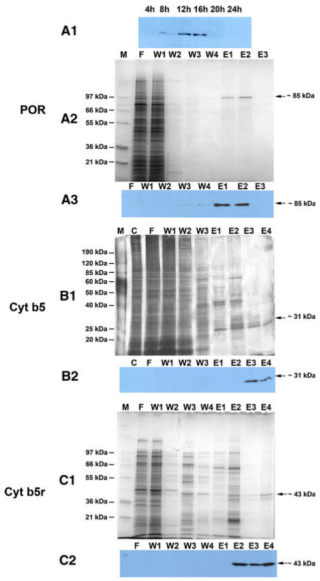

Fig. 4.

Heterologous expression and purification of the *P. chrysosporium* P450 redox proteins POR, cyt b5, and cyt b5r. Top panel: (A1). Temporal expression of the yeast expressed fungal POR as analyzed by Western blot analysis using anti-His antibody; (A2). Gel analysis of the sequential purification steps for the yeast expressed fungal POR. Ten microliter of the flowthrough (F), 20 μl each of the washes (W1–W4) and 30 μl each of the eluates (E1–E3) were loaded onto a 10% SDS-PAGE gel and the bands were visualized by Coomassie blue staining; (A3). Western blot analysis of the above POR purification analysis gel using anti-His antibody. Middle panel: Heterologous expression and purification of the *P. chrysosporium* cyt b5 in *E. coli*. (B1). Gel analysis of the sequential purification steps for the *E. coli* expressed fungal cyt b5. Gel separation of the purification fractions was performed as described above and the extent of purification assessed using silver stain; (B2). Western blot analysis of the above cyt b5 purification analysis gel using anti-His antibody. Bottom panel: Heterologous expression and purification of the *P. chrysosporium* cyt b5r in *E. coli*. (C1). Gel analysis of the sequential purification steps using same conditions as above and the extent of purification assessed by Coomassie blue staining; (C2). Western blot analysis of the above cyt b5r purification analysis gel using anti-His antibody

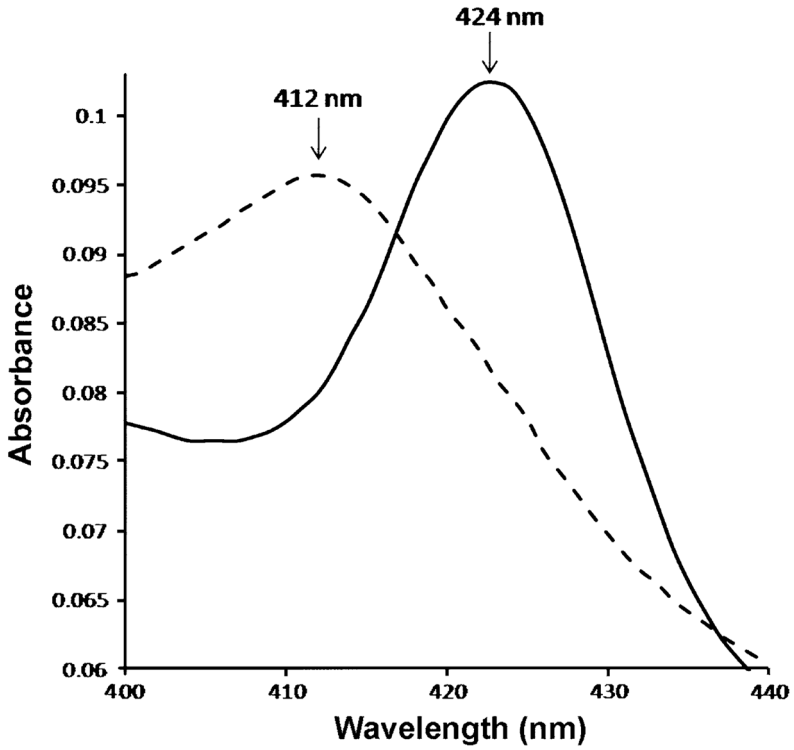

Fig. 5.

Typical absorbance spectra of the purified recombinant cytochrome b5 (cyt b5) of *P. chrysosporium*. A defined amount of the purified cyt b5 protein (equivalent to 1.212 nmol) was suspended in 200 μl of 50 mM Tris–HCl, pH 8.0 in individual wells of a microtiter plate and absorbance spectra were recorded for the air-oxidized (*dashed line*) and sodium dithionite-reduced (*solid line*) forms of cyt b5. Characteristic spectra with maximum absorption wavelength for oxidized (412 nm) and reduced (424 nm) cyt b5 are shown

Table 1

Structural analysis of the isolated P450 redox proteins in *P. chrysosporium*

Gene	Introns	Intron sizes (bp)	cDNA size (bp)	GC content (°C)	Predicted mol. wt. (kDa)	Observed mol. wt. (kDa)
POR	3[a]	62[a]	2211[a]	56.1	81.7	~85 kDa (with His tag)
		50[a]				
		58[a]				
cyt b5	2	51	717	60.5	25.38	~31 kDa (with His tag)
		60				
cyt b5r	3	56	966	58.8	35.52	~43 kDa (with His tag)
		61				
		470				

[a]Data obtained in our earlier study (see reference [14])

Table 2

Specific activity, yield, and fold purification for the heterologously expressed fungal P450 oxidoreductase (POR) and cytochrome b5 reductase (cyt b5r) proteins

Recombinant protein	Form	Total volume (ml)	Protein content (mg/ml)	Total protein (mg)	Substrate for reduction	Activity (U/ml)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold- purification
POR	Crude extract	2.6	9.7	25.1	cyt c	119	310[a]	12.3	100	1
	Purified protein	6	0.1	0.5	cyt c	8.3	49.5[a]	91.7	15.9	7.4
cyt b5r	Crude extract	6	6.63	39.8	cyt b5**	12.2	72[b]	1.84	100	1
	Purified protein	0.125	0.01	0.001	cyt b5**	11.4	1.4[b]	1266.67	1.98	688.57

[a] POR activity was determined based on its ability to reduce cytochrome c using NADPH as the electron donor by monitoring the increase in absorbance at 550 nm. The extinction coefficient used for the calculation was 21 mM^{-1} cm^{-1}

[b] Activity of cyt b5r was determined based its ability to reduce the *E. coli* expressed and purified *P. chrysosporium* cyt b5 (**) using NADH as the electron donor by monitoring the increase in absorbance at 424 nm. The extinction coefficient used for the calculation was 100 mM^{-1} cm^{-1}