Identification of an NADH-Cytochrome b\textsubscript{5} Reductase Gene from an Arachidonic Acid-Producing Fungus, \textit{Mortierella alpina} 1S-4, by Sequencing of the Encoding cDNA and Heterologous Expression in a Fungus, \textit{Aspergillus oryzae}

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Based on the sequence information for bovine and yeast NADH-cytochrome \textit{b}_5 reductases (CbRs), a DNA fragment was cloned from \textit{Mortierella alpina} 1S-4 after PCR amplification. This fragment was used as a probe to isolate a cDNA clone with an open reading frame encoding 298 amino acid residues which show marked sequence similarity to CbRs from other sources, such as yeast (\textit{Saccharomyces cerevisiae}), bovine, human, and rat CbRs. These results suggested that this cDNA is a CbR gene. The results of a structural comparison of the flavin-binding \textit{β}-barrel domains of CbRs from various species and that of the \textit{M. alpina} enzyme suggested that the overall barrel-folding patterns are similar to each other and that a specific arrangement of three highly conserved amino acid residues (i.e., arginine, tyrosine, and serine) plays a role in binding with the flavin (another prosthetic group) through hydrogen bonds. The corresponding genomic gene, which was also cloned from \textit{M. alpina} 1S-4 by means of a hybridization method with the above probe, had four introns of different sizes. These introns had GT at the 5′ end and AG at the 3′ end, according to a general GT-AG rule. The expression of the full-length cDNA in a filamentous fungus, \textit{Aspergillus oryzae}, resulted in an increase (4.7 times) in ferricyanide reduction activity involving the use of NADH as an electron donor in the microsomes. The \textit{M. alpina} CbR was purified by solubilization of microsomes with cholic acid sodium salt, followed by DEAE-Sephaloc, Mono-Q HR 5/5, and AMP-Sepharose 4B affinity column chromatographies; there was a 645-fold increase in the NADH-ferricyanide reductase specific activity. The purified CbR preferred NADH over NADPH as an electron donor. This is the first report of an analysis of this enzyme in filamentous fungi.

The filamentous fungus \textit{Mortierella alpina} 1S-4, belonging to the Zygomycetes, is used industrially to produce arachidonic acid (40); the latter is converted to eicosanoids (e.g., prostaglandins, leukotrienes, and thromboxanes), which are essential bioactive compounds (30, 41). However, the mechanism of available polyunsaturated fatty acids (PUFAs) in oil bodies is under study. Desaturase-defective mutants are able to accumulate various \textit{ω}-3 fatty acids (40); the latter is converted to eicosanoids (e.g., prostaglandins, leukotrienes, and thromboxanes), which are essential bioactive compounds (30, 41). However, the mechanism of available polyunsaturated fatty acids (PUFAs) in oil bodies is under study. Desaturase-defective mutants are able to accumulate various \textit{ω}-3 fatty acids (40); the latter is converted to eicosanoids (e.g., prosta-

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also a soluble form of CbR that reacts with a soluble form of cytochrome \textit{b}_5 (34). Recent studies have suggested that the soluble and membrane-bound forms of CbR from erythrocytes are encoded by the same gene (36).

It is known that CbR is composed of flavin adenine dinucleotide (FAD)-binding domain and an NADH-binding domain and that a “hinge” \textit{β}-sheet structure connects the two domains (2). A structural comparison of the flavin-binding \textit{β}-barrel domain of CbR and those of other flavin-dependent reductases (e.g., ferredoxin-NADP\textsuperscript{+} reductase [17], phthalate dioxygenase reductase [6], and nitrate reductase [25]) indicated that the overall barrel-folding patterns are similar and that a specific arrangement of three amino acid residues (i.e., Arg, Tyr, and Ser or Thr) is usually necessary for flavin binding (31).

As representative sources of microbial CbRs, two types of CbR have been reported. One is CBR, which is a putative CbR exhibiting considerable similarity to both plant nitrate reductases and mammalian CbRs and which has not been examined with regard to localization in cells; in addition, CBR is essential for life in \textit{Saccharomyces cerevisiae}, as demonstrated by CBR disruption experiments (7). The other is MCR1, which is localized in mitochondria in two forms; one is anchored to the outer membrane, and the other is inserted by digestion at the N-terminal membrane-bound domain (11). However, the functions of the two types of MCR1 in mitochondria are unclear. Thus, while mammalian CbR studies have significantly progressed, there is still a need for further investigation of microbial CbRs.

In mammalian microsomes, there are two electron transport pathways: an NADH-linked system consisting of CbR, cyto-
chrome β, and fatty acid desaturase and an NADPH-linked system consisting of NADPH-cytochrome P-450 reductase and cytochrome P-450 (45). Cytochrome β, is believed to play a role in the NADPH-dependent oxidation of xenobiotics, serving as a donor of the second electron to oxygenated cytochrome P-450 (24). In the presence of transition metals such as iron, microsomes catalyze the production of a variety of potent oxidizing species (which can initiate lipid peroxidation and promote oxidation of hydroxyl radical agents), resulting in the inactivation of enzymes (48). CbR activity is also required for NADH and ferric-ADP-catalyzed lipid peroxidation in rat liver microsomes.

Although CbRs from mammals have been studied genetically, enzymatically, and structurally, there has been no report on a CbR from a filamentous fungus. We focused on CbR as a component of the fatty acid desaturation system in microsomes (21). Here, we describe the isolation and characterization of the CbR gene from M. alpina 1S-4 and the purification of the CbR expressed in Aspergillus oryzae.

MATERIALS AND METHODS

Enzymes and chemicals. The restriction endonucleases and other DNA-modifying enzymes were obtained from Takara Shuzo Co., Ltd., Tokyo, Japan, and Toyobo Co., Ltd., Osaka, Japan. [α-32P]dCTP (110 TBq/mmol) was obtained from Amersham Japan, Tokyo, Japan. All other chemicals were of the highest purity commercially available.

Strains, plasmids, and growth media. For cloning of the CbR gene, M. alpina 1S-4 (AKU 3998; Faculty of Agriculture, Kyoto University, Kyoto, Japan) was used. A. oryzae (niaD) lacking nitrate reductase activity for nitrate assimilation and derived from the wild-type strain RIB90 (29) was used as a recipient in transformation experiments. Escherichia coli JM109 (39) and DH5α (12) and plasmid vector pBluescript II (Stratagene, La Jolla, Calif.) were used for DNA manipulation, unless otherwise specified. pNGA142 is a shuttle vector carrying the ampicillin resistance gene (for selection in E. coli) and the niaD gene of A. oryzae (for nitrate prototrophy selection in A. oryzae) (9). This plasmid also carries the promoter region of the glucoamylase gene (glaA) of A. oryzae flanking unique HindIII and XbaI cloning sites (13, 29).

M. alpina 1S-4 was cultured in a medium (GY medium) containing 20 g of glucose and 10 g of yeast extract (pH 6.0) per liter. The minimal and complete medium consisted of 3% sucrose, 0.2% potassium dihydrogen phosphate, 0.15% magnesium sulfate, 0.05% potassium chloride, 0.05% magnesium sulfate heptahydrate, and iron(II) sulfate heptahydrate (pH 5.5) and dextrose-yeast medium (consisting of 2% dextrose, 1% peptone, and 0.5% yeast extract [pH 5.8], respectively).

PCR amplification and sequence analysis. Two highly degenerate primers were synthesized for the screening of the cDNA and the genomic gene of the M. alpina CbR by means of PCR with the following primers: a sense primer, 5′-GG(G/A)T(T/C)GG(A/G/C/T)CA(A/G)CA(T/C)TAT-3′ (fully degenerate relative to the tryptophan, tyrosine amino acid sequence), GLP [G(T/A)Q], and an antisense primer, 5′-CAT(G/T)G(G/C/T)CA(T/A)ATATCAC(AG/G/T)GTC(G/C)ATACAT-3′ (fully degenerate relative to the conserved amino acid sequence, MAP(G/T)G(T/G)GAM), where I indicates inosine or isoleucine. These primers were used in a Perkin-Elmer Cetus DNA thermal cycler with a final volume of 1 ml. One unit of CbR activity was defined as the amount causing a 1 cm decrease in the optical density at 600 nm with a reaction mixture containing 3 M NADH and 10 M DCPIP, and enzyme in 2 ml of 0.1 M Tris-acetate buffer containing 1 M EDTA and 20% glycerol (pH 8.0) with a Dyno-mill for 15 min. The resultant cell extract was centrifuged at 10,000 × g for 20 min, and the precipitate was discarded. The supernatant was carefully pipetted off, and the loosely packed precipitate was suspended in a half volume of the same buffer and recentrifuged in the same way.

Solubilization and purification of CbR. Unless otherwise specified, all procedures were carried out at 4°C. Washed microsomes were suspended in an equal volume of buffer A containing 20% cholic acid sodium salt. The suspension was incubated at 4°C with reciprocal shaking overnight and then centrifuged at 100,000 × g for 90 min. The resultant supernatant was used for further purification (22). The solubilized supernatant (20 ml) was applied to a Mono-Q HR 5/5 column (Pharmacia, Uppsala, Sweden) previously equilibrated with buffer B (20 mM Tris-acetate, 1 mM EDTA, 20% glycerol, 0.05% cholic acid sodium salt). The column was washed with 200 ml of buffer B and then eluted with a linear concentration gradient of 0 to 1 M NaCl in buffer C. The active fractions (30 ml) were combined, dialyzed against 5 liters of buffer B, and then applied to a Mono-Q HR 5/5 column (Pharmacia) equilibrated with buffer B and then loaded onto an AMP-5 Sepharose 4B column (1 by 5 cm; Pharmacia) equilibrated with buffer C. The column was washed with 20 ml of buffer C and then eluted with a linear concentration gradient of 0 to 0.5 M NaCl in buffer C.

Assay of CbR activities. CbR activity was measured at each purification step by determining the rate of NADH-dependent ferricyanide reduction (43). NADH-dependent ferricyanide reduction was carried out with 0.1 M potassium phosphate buffer (pH 7.5) containing 10−3 M NADH and 10−3 M ferricyanide in a final volume of 1 ml. One unit of CbR activity was defined as the amount causing the reduction of 1 μmol of ferricyanide per min. The activity of CbR with ferricyanide was assayed by measuring the change in the intensity of the absorbance at 420 nm. An extinction coefficient of 1.02 mM−1 cm−1 was used for ferricyanide.

NADH-2,6-dichlorophenol-indophenol (DCPIP) reductase activity was assayed by measuring the change in the optical density at 600 nm with a reaction mixture containing 10−3 M NADH, 10−3 M DCPIP, and enzyme in 2 ml of 0.1 M potassium phosphate buffer (pH 7.5). The molar extinction coefficient of the oxidized dye at 600 nm was assumed to be 21.0 mM−1 cm−1 (40).

Protein determination and gel electrophoresis. The protein was determined by the method of Lowry et al. (34). SDS-PAGE was performed with a gel containing 12.5% acrylamide and 0.1% SDS and stained with Coomassie brilliant blue G-250. The molecular mass of CbR was estimated by SDS-PAGE with molecular mass markers (Bio-Rad) and calculated to be 57,000. Following electrophoresis, gels were stained and scanned with an HP ScanJet 3c scanner.
N-terminal amino acid sequence analysis. The target bands of cell extracts separated by SDS-PAGE were directly electroblotted onto a polyvinylidene difluoride membrane (Bio-Rad) by a standard procedure (27). After equilibration of the membrane and the acrylamide gel in a transfer buffer containing N-cyclohexyl-3-aminopropanesulfonic acid, the target protein was transferred to a polyvinylidene difluoride membrane by means of an electroblotting system (Typ-Nr. B 33; Biometra, Göttingen, Germany). The electroblotted membrane was used directly for N-terminal sequence analysis by automatic Edman degradation with an Applied Biosystems model 476A amino acid analyzer.

Southern blot analysis. Southern blotting of the restriction endonuclease-digested total DNA was performed essentially as described by Sambrook et al. (39). The 1.2-kb XhoI-SphI fragment from the M. alpina CbR genomic gene or the 1.1-kb M. alpina CbR cDNA digested with EcoRI was labeled with [γ-32P]dCTP by use of a Multiprime DNA labeling system (Amersham, Buckinghamshire, United Kingdom) and then used to probe the Southern blots. Prehybridization and hybridization were performed at 42°C at a high stringency with a solution consisting of 50% (vol/vol) formamide, 2× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate), 1% (wt/vol) SDS, and 10% (wt/vol) dextran sulfate. The blots were then washed twice in 2× SSC for 5 min at room temperature, twice in 2× SSC containing 1% SDS for 20 min at 42°C, and twice in 0.2× SSC containing 1% SDS for 20 min at 42°C.

Nucleotide sequence accession number. The DDBJ accession numbers for the M. alpina CbR gene are AB020034 (cDNA) and AB020035 (genomic gene).

RESULTS

Cloning of the M. alpina CbR cDNA. Two conserved amino acid sequences found in the bovine (33) and yeast (7) CbRs were used as the basis for the design of two primers for PCR. The sequence GLP(V)/V/GQHI (amino acid positions 71 to 78 in bovine CbR and 108 to 115 in yeast CbR) was chosen for the 5′ primer, while the sequence MIAGGTGI(A/T)PM (positions 176 to 186 in bovine CbR and 189 to 199 in yeast CbR) was chosen for the 3′ primer.

A 350-bp fragment was amplified by PCR with these primers and M. alpina 1S-4 genomic DNA as a template. The amplified fragment was subcloned into pCR2.1 to construct pMCR1, and the nucleotide sequence of the latter was determined. The deduced amino acid sequence showed high similarity to those of the bovine and yeast CbRs. With this 32P-labeled 350-bp fragment as a probe, an M. alpina CbR cDNA was isolated from a λgt10 cDNA library. A KpnI fragment derived from the λgt10 clone was subcloned into pBluescript II, and its nucleotide sequence was determined. This KpnI fragment contained an open reading frame which is predicted to encode a protein of 298 amino acids with a molecular mass of 32 kDa.

Cloning of the M. alpina 1S-4 CbR genomic gene. The genomic cosmid library of M. alpina 1S-4, which was constructed at the BamHI sites of the SuperCos1 cosmid vector (Stratagene), was screened for a CbR genomic gene with the above 350-bp fragment as a probe. Several clones were obtained from about 3,000 colonies. One of these clones had a 38-kb DNA fragment including the M. alpina CbR gene. A 3.3-kb DNA fragment was obtained from this cosmid clone by SphI digestion, and its sequence was determined. The first ATG codon at nucleotide 378 from the SphI site was assumed to be the translation initiation codon. This conclusion was based on the following observations: (i) no other in-frame ATG codon was found upstream from the presumed N-terminal methionine residue, and (ii) the predicted N-terminal amino acid sequence upstream from this methionine residue exhibits no similarity to those of the bovine (33) and yeast (7) CbRs. Sequences similar to a TATA box and a CAAT box, which are generally present in the promoter regions of eukaryotes, were not observed. The cDNA had a G+C content of 54.9% and showed a strong preference for C at the third position of codons (data not shown). This gene had four introns (117, 116, 100, and 98 bp) which conformed to the GT-AG rule with regard to RNA splicing.

Comparison of the CbR sequence of M. alpina 1S-4 with those of other organisms. The deduced amino acid sequence of the cloned M. alpina CbR cDNA exhibited 50 and 35% identity with the S. cerevisiae sequence (7, 11) and the mammalian sequence (33, 46, 49), respectively (Fig. 1). On calculation of the hydrophobic index by the Kyte-Doolittle method, the N-terminal domain of the M. alpina CbR was found to be highly hydrophobic (data not shown). This finding suggests that the M. alpina CbR is a membrane-bound protein, anchored through the N-terminal domain. Like nitrate reductases, in general, CbRs have FAD-binding domains as part of an electron transfer system. The M. alpina CbR also exhibited significant similarity (32% identity) to the FAD-binding domain of nitrite reductases (25).

In particular, high similarity between M. alpina and other organisms was observed in both the FAD-binding and the NADH-binding domains. Although the predicted β-sheet structure, which connects the two domains, is conserved in mammalian CbRs, the M. alpina and S. cerevisiae CbRs lack such a β-sheet structure. This finding suggests that it is possible for the M. alpina CbR to be folded closely in connection with the two domains. The specific arrangement of the three amino acid residues (i.e., Arg, Tyr, and Ser) which are usually necessary for flavin binding is conserved in the M. alpina CbR.

Genomic Southern blot analysis. The genomic DNA of M. alpina was analyzed by Southern blot hybridization with the M. alpina CbR genomic gene and the CbR cDNA as probes. As shown in Fig. 2, a single positive band was observed in each lane in which the XhoI-, SphI-, and EcoRI-digested genes were loaded. The signal obtained with the genomic gene as a probe showed strong intensity. As the CbR genomic gene contained four introns, the signal obtained with the CbR cDNA (which did not contain introns) as a probe showed weak intensity. This finding demonstrated that only one CbR gene existed on the M. alpina genomic DNA. This finding also supported the view that the gene would be involved in arachidonic acid biosynthesis.

Expression of the M. alpina CbR cDNA in A. oryzae. The M. alpina CbR cDNA was ligated to the fungal expression vector pNGA142, resulting in the construction of a plasmid designated pMCR30. After transformation, several transformants were obtained on selective medium plates as described in Materials and Methods. To examine the expression of the M. alpina CbR in A. oryzae, the ferricyanide reduction activity in transformants cultured in 300-ml Erlenmeyer flasks containing 50 ml of maltose medium at 30°C for 3 days was analyzed as described in Materials and Methods. We selected one recombinant, MACR-1, which exhibited high ferricyanide reduction activity; the other transformants exhibited similar properties. The ferricyanide reduction activity (5.07 U/mg) in the microsomal fraction of MACR-1 was 4.7 times higher than that (1.08 U/mg) in the same fraction of the control strain containing only pNGA142 without any insert DNA. When we made microsomes from the cells for the purification of the M. alpina CbR (Table 1), the activity was less than 5.07 U/mg (1.69 U/mg). We believe that these values were influenced by different conditions of agitation and aeration.

Purification and characterization of the M. alpina CbR expressed in A. oryzae. Although Triton X-100 solubilized most of the CbR, it was difficult to remove excess Triton X-100 from the solubilized solution; moreover, most of the solubilized CbR appeared to undergo aggregation. So, we tried to find a detergent that could replace Triton X-100 among 16 kinds of detergents and selected cholic acid sodium salt to stabilize the expressed CbR during the purification procedure. It was easy
to remove extra cholic acid sodium salt from samples by dialysis because of the low molecular mass of the cholic acid sodium salt aggregate.

The purification of the *M. alpina* CbR is summarized in Table 1. Glycerol was necessary throughout the purification to maintain the enzyme activity, except during AMP-Sepharose 4B column chromatography. A purification of 645 fold and a yield of 0.057% were obtained from microsomes of MACR-1 containing the *M. alpina* CbR cDNA, as monitored by NADH-ferricyanide reductase activity. The CbR eluted from the AMP-Sepharose 4B column was found to be homogeneous, as judged by SDS-PAGE (Fig. 3). In particular, AMP-Sepharose 4B column chromatography was effective for the purification of CbR. The molecular mass determined for the purified CbR by SDS-PAGE analysis was 33,000 Da. The determined N-terminal 10-amino-acid sequence of the purified CbR was consistent with the putative sequence derived from the *M. alpina* CbR cDNA; the purified CbR would be derived from the introduced gene.

Table 2 shows the specific activities of the *M. alpina* CbR with two types of electron donors and acceptors. The mammalian CbRs have been reported to be specific for NADH. The *M. alpina* CbR showed the same specificity, being capable of reducing ferricyanide and DCPIP in the presence of NADH.

![FIG. 1. Comparison of the deduced amino acid sequences of *M. alpina* 1S-4, yeast, bovine, human, and rat CbRs. The alignment was performed by introducing gaps (hyphens) to achieve maximum homology with the GENETYX-MAC 7.0 program (Software Development Co., Ltd., Tokyo, Japan). Conserved residues are shown in white. The three amino acid residues needed for flavin binding are indicated by asterisks.](http://aem.asm.org/)

<table>
<thead>
<tr>
<th>M. alpina 1S-4</th>
<th>Yeast (CBR)</th>
<th>Bovine</th>
<th>Human</th>
<th>Rat</th>
</tr>
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**Table 1.** Comparison of the deduced amino acid sequences of *M. alpina* 1S-4, yeast, bovine, human, and rat CbRs. The alignment was performed by introducing gaps (hyphens) to achieve maximum homology with the GENETYX-MAC 7.0 program (Software Development Co., Ltd., Tokyo, Japan). Conserved residues are shown in white. The three amino acid residues needed for flavin binding are indicated by asterisks.
but not in the presence of NADPH. The specific activities of the M. alpina CbR were the same as those of the rabbit CbR reported previously (47).

**DISCUSSION**

The industrial strain M. alpina 1S-4 accumulates unsaturated C₁₈ and C₂₀ fatty acids in its membrane and lipid bodies (15, 16, 38, 40). A Δ5-desaturase gene was very recently isolated from M. alpina 1S-4 CBS 210.32, and expression of the gene in a yeast, S. cerevisiae, resulted in the accumulation of arachidonic acid with the exogenous addition of dihomo-γ-linolenic acid (28). Moreover, expression of the M. alpina Δ5-desaturase cDNA in transgenic canola seeds was very recently reported (20). Therefore, the M. alpina genes involved in the biosynthesis of PUFA s have received increasing attention not only in academic but also in applied areas.

As a component of the microsomal electron transport system, CbR is involved in the desaturation and elongation of fatty acids (18, 32), cholesterol biosynthesis (37), and drug metabolism (35). In the biosynthesis of PUFA s in M. alpina, CbR should be a key enzyme swiftly facilitating the desaturation and elongation of PUFA s. Here, we isolated the CbR cDNA and the CbR genomic gene from M. alpina 1S-4 by using a degenerate PCR approach based on the amino acid sequence motifs conserved in other microsomal CbRs. To our knowledge, this is the first report of the cloning of a gene encoding a CbR from a filamentous fungus.

The genomic gene encoding CbR has four introns, of 117, 116, 100, and 98 bp. All four introns conform to the GT-AG rule with regard to RNA splicing and exhibit no homology with one another. The sequence encoding CbR has a G+C content of 54.9%, whereas the introns have G+C contents of 44.4, 41.2, 47.0, and 39.8%. These data are consistent with the general rule that a sequence in an intron comprises much more A and T than G and C.

The M. alpina CbR has a typical structure of a CbR: (i) a monomer of the CbR comprises 298 amino acid residues, which corresponds to the general lengths of other CbRs; (ii) the possible FAD-binding domain at the N terminus and the possible NADH-binding domain at the C terminus exhibit high sequence similarity with those of other CbRs; (iii) the specific arrangement of the three amino acid residues (i.e., Arg, Tyr, and Ser) which are predicted to be essential for flavin binding in other enzymes (31) is observed in the M. alpina CbR; and (iv) a series of amino acid sequences at the N terminus shows strong hydrophobicity and therefore may bind the membrane of the endoplasmic reticulum. In the CbR of M. alpina, however, there is no β-sheet structure, which is known to connect the FAD-binding domain and the NADH-binding domain in mammalian CbRs. Unlike mammalian CbRs, the microbial CbRs derived from M. alpina and S. cerevisiae might have evolved independently.

CbR, which receives an electron from NADH and then donates it to cytochrome b₅, is a major component of the microsomal electron transport system in eukaryotes (18, 32, 35, 37). However, a mutant lacking CbR activity shows that the activity can be replaced by another electron transporter. For mammals, it has been verified that CbR can be substituted by cytochrome P-450 reductase in vitro (8, 19, 32), and disruption of the yeast gene encoding cytochrome P-450 reductase does not entirely abolish cytochrome P-450 reductase activity (42). Furthermore, cell viability is not affected by deletion of the cytochrome P-450 reductase gene, although such deletion

**TABLE 1. Purification of the M. alpina CbR expressed in A. oryzae**

<table>
<thead>
<tr>
<th></th>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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<td>645</td>
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FIG. 2. Genomic Southern blot analysis of M. alpina 1S-4 with the CbR genes as probes. Total DNA (4 μg) from M. alpina was digested with three endonucleases (sites not present in the M. alpina CbR gene), separated by electrophoresis on a 1.0% agarose gel, transferred to a nylon membrane, and hybridized with the labeled 1.2-kb BglII fragment from the M. alpina CbR genomic gene (A) or the labeled 1.1-kb M. alpina CbR cDNA digested with EcoRI (B).

**FIG. 3. SDS-PAGE of the purified M. alpina CbR.** Row A, purified enzyme; row B, molecular mass standards—phosphorylase (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).
leads to a marked reduction in the growth rate (42). Double disruption of the yeast CbR and cytochrome P-450 reductase genes leads to a loss of viability, suggesting that indeed one can compensate for the loss of the other.

In S. cerevisiae, the genes for two types of CbR have been cloned. One is CBR, which is a novobiocin-binding protein of unknown location, and the other is MCR1, which is located in mitochondria. The functions of these CbRs remain unknown. The M. alpina CbR exhibits the highest homology to the yeast CBR (50% identity) and contains a hydrophobic N-terminal region. Considering these findings, the M. alpina CbR may be located in the membrane of the endoplasmic reticulum but not in mitochondria.

We succeeded in expressing the M. alpina CbR gene in a filamentous fungus, A. oryzae, although we failed to express it in E. coli. This result is likely to be due to differences in codon usage between eukaryotes and prokaryotes and/or hydrophobicity. The ferricyanide reduction activity in the microsome fraction of MACR-1 was 11.3 times higher than that in the cytosol fraction of MACR-1, suggesting that the expressed CbR is incorporated into the endoplasmic reticulum of MACR-1 and functions as an electron transporter. Although, of course, the host A. oryzae contains its own CbR, the ferricyanide reduction activity in the microsomes of MCR-1 was 4.7 times higher than that of the control strain. During purification of the recombinant CbR, most of the CbR activities were lost in the DEAE-Sephacel step. Although most of the CbR was solubilized with the detergent (cholic acid), a portion of CbR was solubilized in the form of aggregates with other membrane-bound proteins. After the DEAE-Sephacel step, the CbR activities were detected in many fractions; the protein peak with activity was broad. Only selected fractions with higher activities were pooled. We also found that the major loss of CbR activities resulted in difficulty with the purification.

We tried to purify a CbR from M. alpina, but we failed, perhaps for two reasons. (i) Because the cell wall of M. alpina is very rigid, it was difficult to disrupt. (ii) The microsomes containing the CbR of M. alpina were too small to use for purification (data not shown). Therefore, we could not compare protein and enzymatic characteristics between the recombinant CbR from A. oryzae and the authentic CbR from M. alpina.

Here, we succeeded in high-level heterologous expression in a filamentous fungus, A. oryzae, and purification of the CbR expressed in A. oryzae. For this reason, a gene derived from a filamentous fungus is likely to be expressed in a heterologous filamentous fungal host. We also found that the purified CbR used NADH but not NADPH as an electron donor; when NADH was used as the electron donor, DCPIP was found to be reduced by the purified CbR, the specific activity being 114 µmol/min/mg. These findings also support the notion that the cDNA cloned here is definitely a CbR gene.

Further characterization of the CbR is required. For example, determination of the molecular mass of the CbR, determination of whether or not the CbR is able to donate an electron to cytochrome b5, and a comparison of protein and enzymatic characteristics between the recombinant CbR from A. oryzae and the authentic CbR from M. alpina are potential areas of study.

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REFERENCES


* ND, not detected.


