

Subcellular localization of glyoxylate cycle key enzymes involved in oxalate biosynthesis of wood-destroying basidiomycete *Fomitopsis palustris* grown on glucose

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This study investigated the subcellular localization of key enzymes of the glyoxylate cycle, i.e. isocitrate lyase (ICL; EC 4.1.3.1) and malate synthase (EC 2.3.3.9), that function constitutively in coordination with oxalate biosynthesis of glucose-grown *Fomitopsis palustris*. The ICL purified previously from *F. palustris* is termed FPICL1. Subcellular fractionation analysis of the cell homogenate by the sucrose density-gradient method showed that both key enzymes were present in peroxisomes, whereas acetyl-CoA synthase (EC 6.2.1.1) and oxalate-producing oxaloacetate acetylhydrolase (EC 3.7.1.1) were cytosolic. The peroxisomal localization of FPICL1 was further confirmed by electron microscopic and immunocytochemical analysis with anti-FPICL1 antibody. In addition, the peroxisomal target signal, composed of SKL at the C terminus of the cDNA encoding FPICL1, was found, which also suggests that FPICL1 is peroxisomal. Accordingly, it is postulated that transportation of succinate from peroxisomes to mitochondria, and vice versa, for the transportation of isocitrate or citrate, occurs in glucose-grown *F. palustris* for the constitutive metabolic coordination of the TCA and glyoxylate cycles with oxalate biosynthesis.

INTRODUCTION

The glyoxylate (GLOX) cycle, which was first discovered by Kornberg & Krebs in 1957, involves two key enzymes, isocitrate lyase (ICL; EC 4.1.3.1) and malate synthase (MS; EC 2.3.3.9). ICL, *threo*-D₅-isocitrate-glyoxylate-lyase, catalyses the reversible conversion of isocitrate to succinate and glyoxylate (Campbell *et al.*, 1953). The glyoxylate intermediate is further condensed with acetyl-CoA, yielding malate by catalysis of MS (Wong & Aji, 1956). Thus,

for maintaining the TCA cycle, the GLOX cycle enables a net synthesis of C₄ carboxylic acids from a carbon source of C₂ compounds (Kornberg & Madsen, 1957; Kornberg & Krebs, 1957; Kornberg, 1966), such as acetate, ethanol, and fatty-acid-derived acetyl-CoA.

In general, micro-organisms induce ICL as an adaptive enzyme for gluconeogenesis only when they are grown on C₂ compounds (Vanni *et al.*, 1990). However, when glucose is present together with these non-fermentable compounds, expression of an ICL-encoding gene is repressed, or, in some cases, the ICL protein is further inactivated, which is called catabolite repression or inactivation (Herrero *et al.*, 1985; López-Boado *et al.*, 1988; Fernández *et al.*, 1992; De Lucas *et al.*, 1994; Ordiz *et al.*, 1996; Amor *et al.*, 2000; Schüller, 2003; López *et al.*, 2004).

In contrast to the above biochemical features of ICLs, both ICL and MS occur as constitutive enzymes in a wide variety of glucose-grown wood-destroying basidiomycetes, including *Fomitopsis palustris* (Munir *et al.*, 2001a). Furthermore, *F. palustris* accumulates oxalate in the culture fluid,

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Abbreviations: ACS, acetyl-CoA synthase; DAB, diaminobenzidine; FPICL1, *Fomitopsis palustris* isocitrate lyase 1; GLOX, glyoxylate; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; MS, malate synthase; NTA, nitrilotriacetate; ODH, 2-oxoglutarate dehydrogenase; OXA, oxaloacetate acetylhydrolase; PNS, post-nuclear supernatant; PTS1, peroxisomal target signal 1; SDH, succinate dehydrogenase.

producing a yield of 80% based on the amount of glucose consumed during vegetative growth (Munir *et al.*, 2001b). The acid formed plays a crucial role in the wood-decaying process (Dutton & Evans, 1996; Shimada *et al.*, 1997). Biochemical analysis of the unique oxalate fermentation of *F. palustris* has led to the conclusion that the fungus acquires biochemical energy for growth by oxidizing glucose to oxalate. In such an acid fermentation process, ICL, shared by the GLOX and TCA cycles, plays a major role, whereas neither isocitrate dehydrogenase (IDH) nor 2-oxoglutarate dehydrogenase (ODH) plays a significant role (Munir *et al.*, 2001b). Thus, the constitutive key enzyme ICL replenishes the intermediates glyoxylate and succinate to the GLOX and TCA cycles, respectively (Munir *et al.*, 2001b). The role of *F. palustris* ICL is contrasted with the hither-to-known function of ICL required for gluconeogenesis in other micro-organisms.

In view of this unique carbon metabolism in *F. palustris*, one question arises: in which organelle in the cell of *F. palustris* are the GLOX enzymes cytochemically compartmentalized? Microbial ICL is generally found in microbodies called peroxisomes (Osumi *et al.*, 1978; Valenciano *et al.*, 1996, 1998; Titorenko *et al.*, 1998; Maeting *et al.*, 1999) or glyoxysomes (Kionka & Kunau, 1985), with a few exceptions (Taylor *et al.*, 1996; Chaves *et al.*, 1997; Ono *et al.*, 2003). In the case of basidiomycetes, the ICL of the litter-decomposing *Coprinus lagopus* grown on acetate has been found to occur in peroxisomes by subcellular fractionation analysis (O'Sullivan & Casselton, 1973). However, those authors detected ICL activity from a lighter buoyant density fraction than the mitochondrial fraction, which contradicts results reported for several other micro-organisms. The ICL of *Coprinus cinereus* acu-7 has been proposed to be peroxisomal based on the presence of the peroxisome target signal (PTS1) (Subramani, 1993) at the C terminus (Chaure *et al.*, 1997), without direct experimental evidence. Furthermore, localization of the induced ICLs has been investigated for micro-organisms grown on C₂ compounds, but, to the best of our knowledge, the ICL localization for glucose-grown micro-organisms has not been reported.

Therefore, we were motivated to investigate the intracellular localization of the GLOX cycle enzymes, and other enzymes involved in oxalate fermentation, in glucose-grown *F. palustris*. Previously, we purified and characterized ICL from *F. palustris* (Munir *et al.*, 2002), which is termed FPICL1 in this study. We report here the characterization of FPICL1 cDNA encoding FPICL1, and peroxisomal localization of FPICL1 based on subcellular fractionation and immunocytochemical experiments using anti-FPICL1 antibody. The localization of MS, acetyl-CoA synthase (ACS; EC 6.2.1.1) and oxaloacetate acetylhydrolase (OXA; EC 3.7.1.1) was also determined. The results are discussed in relation to possible transportation of metabolites between subcellular sites, and also in relation to the constitutive metabolic coordination of the TCA and GLOX cycles with the oxalate biosynthesis.

METHODS

Fungal strain and culture conditions. The wood-destroying basidiomycete *F. palustris* (Berkeley et Curtis) Murill (formerly named *Tyromyces palustris*) strain TYP-6137, a Japanese industrial standard fungus for wood-preservative efficacy tests, was used in this study. This fungus was maintained at 32 °C on a potato glucose agar (PDA) culture. The fungal inocula were prepared from a colony fully grown on the agar, and removed with a cork borer (6 mm diameter). Ten plugs of mycelia were used as inocula, and cultured at 32 °C for 4 days in the dark in a 1 litre Erlenmeyer flask, with 200 ml 2% (w/v) glucose medium (pH 5.5) containing 0.8% (w/v) peptone, 0.05% (w/v) KH₂PO₄, 0.05% (w/v) K₂HPO₄, 0.03% (w/v) MgSO₄·7H₂O and 5 p.p.m. thiamine/HCl (Munir *et al.*, 2001a). Fresh mycelia were processed for the experiments described below.

Amino acid sequencing. Purified ICL protein (approx. 80 µg; Munir *et al.*, 2002) was digested with lysylendopeptidase, and the resulting peptides were separated by HPLC on a reverse-phase C18 column. N-terminal amino acid sequences were determined by use of an ABI Precise 491 automated protein sequencer (Applied Biosystems). Protein concentrations were determined by the method of Bradford (1976), using BSA as a standard.

Isolation of cDNA encoding FPICL1. Total RNA was extracted from the mycelia of glucose-grown *F. palustris* by use of a QuickPrep Total RNA Extraction kit (Amersham Biosciences), according to the manufacturer's instructions. PolyA⁺ RNA was isolated from the total RNA using Oligotex-dT30 <Super> (Takara Bio), and cDNA was synthesized with a Timesaver cDNA synthesis kit (Amersham Biosciences), followed by size fractionation into pools containing more than 0.5 kb. The cDNA pool was cloned into a lambda ZAP II vector containing the whole phagemid pBluescript SK sequence (Stratagene), and packaged into λ phage extract (Gigapack III packaging extract kit; Stratagene). The cDNA library was constructed with 3.5 × 10⁵ p.f.u. µg⁻¹.

In order to prepare probe for the screening of FPICL1, PCR was performed with the cDNA, prepared as described above, as a template, and a pair of primers, (P1, 5'-GGTCACATGGCTGGTAAGGT-3'; and P2, 5'-GACCGCGAGAGTGATGAAC-3'), which were designed from the sequences within the *Aspergillus nidulans* ICL gene (*acuD*; GenBank accession no. X62696). We screened cDNA encoding the putative protein, including the internal amino acid sequence of FPICL1. The amplified fragment was subsequently used as an [α -³²P]dATP-labelled probe for cDNA library screening by standard plaque hybridization procedures (Sambrook *et al.*, 1989). Because the obtained cDNA fragment lacked the 5' region, we performed PCR using a cDNA library that had been newly constructed by Takara Bio as a template, and a pair of primers (P3, 5'-GAAAGAA-AAAAATATACCCAGC-3'; and P4, 5'-ACCTTGCCCGCCATA-TGCCCGCACTTCTT-3'), in order to determine the sequence of the 5' end of the cDNA. DNA sequencing was performed by Big Dye terminator v2.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 377 DNA sequencer (Applied Biosystems).

Subcellular fractionation. Cell organelles were obtained from the protoplast homogenate by differential centrifugation. Approximately 100 g (wet wt) of mycelia were harvested by filtration through cheesecloth, and washed well with distilled water. Mycelial mats were suspended in a lysing buffer [50 mM maleate, pH 6.0, containing 0.6 M mannitol, 1 mM EDTA and 5% (w/v) Lysing Enzyme (Sigma-Aldrich)], and gently shaken at 28 °C. After 2–3 h, protoplast formation was complete, and the lysate was chilled to 4 °C. The protoplasts were collected at 4 °C by a low-speed centrifugation, resuspended in a protoplast buffer (50 mM Tris/HCl, pH 7.5) containing 1 M sorbitol, 1 mM EDTA and 1 mM PMSF, and homogenized in a

Potter-Elvehjem homogenizer with a loosely fitting Teflon pestle. The homogenate was centrifuged at 1500 g for 10 min to remove cell debris and nuclei. The post-nuclear supernatant (PNS) was filtered through Miracloth (Calbiochem), and then centrifuged at 20 000 g for 20 min, to yield pellet (20KgP) and supernatant (20KgS) fractions. The 20KgP fraction, consisting mainly of mitochondria and peroxisomes, was resuspended in a suspension buffer (50 mM Tris/HCl, pH 7.5) containing 0.5 M sucrose, 1 mM EDTA and 1 mM PMSF, and layered onto a discontinuous sucrose density-gradient [1 ml 60% (w/v) sucrose, 2 ml each of 50, 46, 43, and 41% sucrose, and 1 ml 35% sucrose, in 10 mM Tris/HCl (pH 7.5)]. The gradient solution was centrifuged at 10 000 g for 5 h at 4 °C in a Beckman SW40 Ti swinging-bucket rotor. Fractions of 0.75 ml were collected from the bottom of the tube, and analysed for various enzyme activities. The sucrose concentration of each fraction was determined refractometrically at 25 °C. Each operation was done in triplicate.

Enzyme assays. All enzyme assays were performed spectrophotometrically at 30 °C by use of a Shimadzu UV-1650P spectrophotometer. ICL and MS activities of a glyoxylate cycle marker were determined by the method of Dixon & Kornberg (1959). ICL activity was assayed by measurement of the increase in absorbance at 324 nm due to the formation of the phenylhydrazone derivative of glyoxylate produced from isocitrate. MS activity was determined on the basis of the consumption of acetyl-CoA, with a decrease in absorbance at 340 nm. Catalase (EC 1.11.1.6) activity, as a peroxisomal marker, was determined by measuring the decomposition of H₂O₂ by the method of Aebi (1983). Succinate dehydrogenase (SDH; EC 1.3.5.1) activity as a mitochondrial marker was measured on the basis of a modified method of Moore & Ewaze (1976). Malate dehydrogenase (MDH; EC 1.1.1.37) activity was assayed as described by Labrou *et al.* (1997). ACS activity was assayed according to the method of Heather & Fairhurst (1998). OXA activity was determined by measurement of the decrease in absorbance at 255 nm due to the hydrolysis of oxaloacetate (Lenz *et al.*, 1976). One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation or decomposition of 1 µmol product or substrate, respectively, per minute, under the conditions described.

Generation of anti-FPICL antibody. To obtain the pure protein [terminated with an SKL tripeptide representing a peroxisome targeting signal 1 (PTS1)] for generating an anti-FPICL1 antibody, the pET-32 Ek/LIC expression system (Novagen) was used. A DNA fragment encoding C-terminal 336 aa (out of a total of 539 aa) was amplified by PCR with the primer pairs designed from the FPICL1 sequences, and subcloned into the expression vector. The truncated protein is expressed as the fusion protein with thioredoxin, H-tag and S-tag at the N terminus. *Escherichia coli* strain Origami B (DE3) was cultured in Luria-Bertani medium supplemented with 100 µg ampicillin ml⁻¹ at 30 °C, and protein expression was induced by IPTG added at a final concentration of 0.1 mM. Cells were harvested by centrifugation, and suspended in 20 mM Tris/HCl (pH 7.0) containing 0.3 M NaCl and 20 mM imidazole. After sonication, cell lysates were subjected to affinity chromatography using a Ni²⁺-nitrilotriacetate (NTA) column, and the purified fusion protein was incubated with enterokinase to remove the N-terminal tags. The enterokinase coexisting in the sample was captured with EKapture Agarose (Novagen). The recombinant protein without a tag was rescued from the fraction passed through the Ni²⁺-NTA column. SDS-PAGE analysis (Laemmli, 1970) revealed a protein band with an apparent molecular mass of 39 kDa, which corresponded to the deduced size of the FPICL1 truncation (not shown).

The homogeneous protein (1.2 mg), emulsified with the same volume of Freund's complete adjuvant (2 ml total volume), was used to immunize rabbits. This was followed by two additional booster

injections. One week after the second injection, a blood sample was collected to measure the titre of the antibody by ELISA. After the last injection, the rabbit was killed under anaesthesia. Blood was centrifuged at 3000 g for 10 min, and the antiserum fraction was pooled, and stored at -80 °C until use.

Western blotting. Proteins were separated on an SDS-PAGE gel containing 15–25% polyacrylamide, and electrotransferred to PVDF membranes (Immobilon-P; Millipore). The membrane was blocked in 0.5% (w/v) skim milk in PBS-T (0.05% Tween-20 in PBS), and then incubated with rabbit polyclonal antibody raised against FPICL1 (1:3000 dilution in PBS-T) or control rabbit serum (1:2000 dilution in PBS-T). After washing in PBS-T, the membrane was incubated with horseradish-peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (1:5000 dilution in PBS-T). The immunoreactive proteins were detected by chemiluminescence with the ECL detection system (Amersham Biosciences), according to the manufacturer's instructions. Preparation of cell-free extracts from *F. palustris*, and purification of the native enzyme, were performed by the methods of Munir *et al.* (2002).

Electron microscopy and immunocytochemistry. *F. palustris* mycelia grown on glucose medium were pre-fixed in 2.5% (v/v) glutaraldehyde and 1.5% (w/v) paraformaldehyde in 20 mM PIPES (pH 7.2) for 3 h at 4 °C. Pre-fixed samples prepared were washed three times with the buffer, and fixed overnight at 4 °C in 1% (w/v) OsO₄ in 20 mM PIPES (pH 7.2). After dehydration in a graded ethanol series at room temperature, the samples were embedded in Spurr's resin (Spurr, 1969), and serially sectioned on a Reichert ultramicrotome (Ultracut E; Reichert-Jung). Ultrathin sections mounted on nickel grids were stained with uranyl acetate at room temperature, followed by lead citrate staining. For cytochemical staining of catalase, a glutaraldehyde/paraformaldehyde-fixed sample was incubated for 3 h at 4 °C with diaminobenzidine (DAB; 1 mg ml⁻¹) in 100 mM Tris/HCl (pH 10.5), containing 0.1% H₂O₂, before OsO₄-fixation. In control experiments with a catalase inhibitor, 3-amino-1,2,4-triazole, a pre-fixed sample was incubated for 30 min with this chemical, and then transferred to DAB solution.

For immunoelectron microscopy, mycelia were fixed overnight at 4 °C in 0.2% glutaraldehyde and 4% paraformaldehyde in 20 mM PIPES (pH 7.2), containing 0.5 mM CaCl₂. Samples were washed three times with the buffer, dehydrated in a graded ethanol series, and embedded in LR-White resin (London Resin). Ultrathin sections were cut with an ultramicrotome. The sections were blocked in 0.1% BSA in PBS for 30 min at room temperature, and incubated for 1 h with anti-FPICL1 antiserum (1:30 dilution in PBS) or non-immune rabbit serum (1:40 dilution in PBS). After washing in PBS, the sections were incubated for 1 h with goat anti-rabbit IgG antibody conjugated with 15 nm gold particles (1:20 dilution in PBS-T). The sections were washed in distilled water, and then stained with uranyl acetate. After staining, all sections were examined with a JOEL 1200 EX transmission electron microscope at 100 kV.

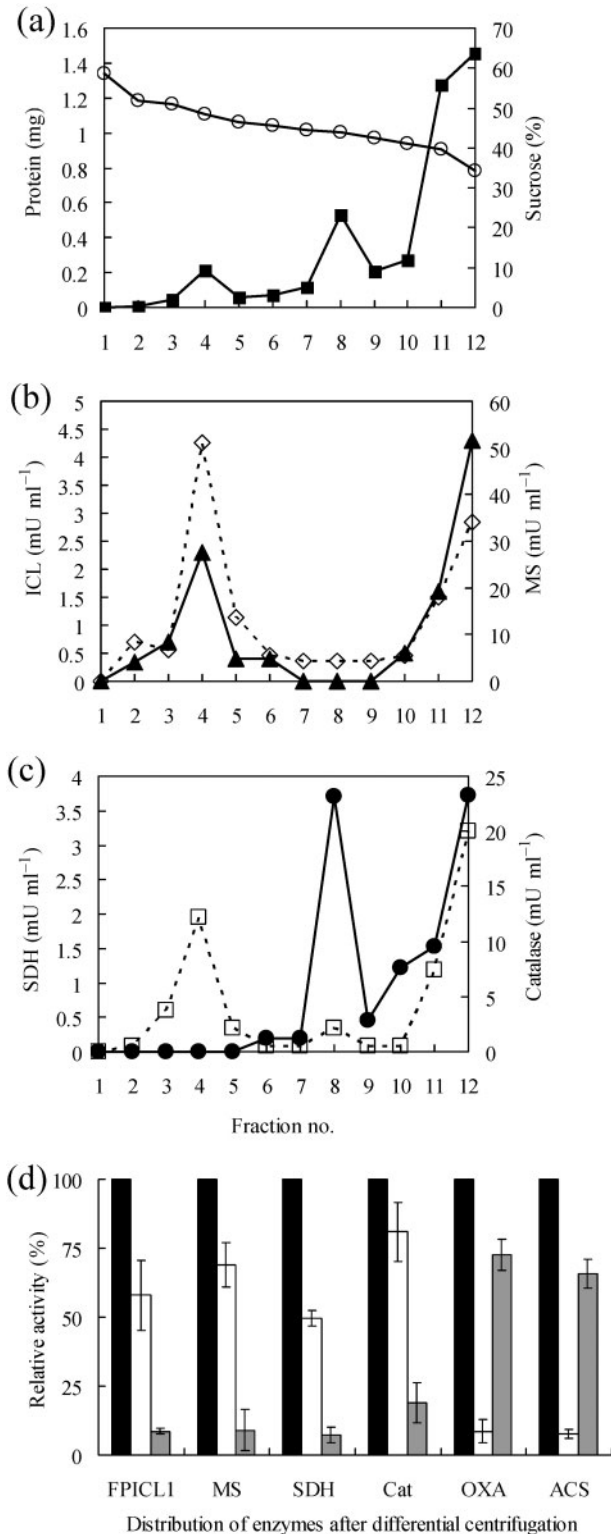
RESULTS AND DISCUSSION

Characterization of FPICL1

We determined the sequence of the cDNA encoding FPICL1 (AB079254). The deduced amino acid sequence contained an ORF that corresponded to 539 residues (Fig. 1). All of the 10 polypeptides obtained from FPICL1 by digestion with lysylendopeptidase were found in this deduced amino acid sequence. The hexapeptide KKCGHM, highly conserved as an ICL signature in the sequences of most ICLs reported to

date, was found in the sequence at aa 201–206. The deduced polypeptide had a predicted molecular mass of 59 735 Da, which is similar to the estimated molecular mass for the purified FPICL1 (Munir *et al.*, 2002). By analogy with acu-D, the ICLs of *A. nidulans* (Gainey *et al.*, 1992), D111,

D165, D167 and E194 are suggested to be a cluster for the Mg²⁺-binding site. The predicted amino acid sequence was compared with those of the ICLs of *Phanerochaete chrysosporium*, *C. cinereus* (Chaure *et al.*, 1997), *A. nidulans* (Gainey *et al.*, 1992) and *Saccharomyces cerevisiae* (Schöler & Schüller, 1993), which revealed 83, 79, 63 and 54 % identity, respectively (Fig. 1). Interestingly, FPICL1 was found to contain the motif KRGT at positions 39–42. This is a putative cAMP-dependent protein kinase phosphorylation site for the T residue. A similar site has been found in *C. cinereus* acu7 (Chaure *et al.*, 1997), and ICL1 sequences of *S. cerevisiae* (Fernández *et al.*, 1992) and *Candida tropicalis* (Atomi *et al.*, 1990). In particular, *S. cerevisiae* ICL1 has been proposed to be phosphorylated at T53, followed by inactivation in the presence of glucose (López-Boado *et al.*, 1988; Ordiz *et al.*, 1996). However, the role of the motif KRGT in FPICL1 remains to be assessed. The C-terminal amino acids SKL, representing PTS1 (Subramani, 1993), suggest a peroxisomal localization of FPICL1 in *F. palustris*.



The localization of catalase in peroxisomes in *F. palustris*

We found that catalase was associated with a type of organelle that was thought to be a peroxisome. The detection of separate activities of SDH and catalase by subcellular fractionation showed that catalase was not localized in the mitochondria (Fig. 2a, c). Furthermore, greater than 80 % of catalase activity was recovered from the pellet fraction (20KgP) containing mitochondria and peroxisomes, whereas 20 % was from the supernatant fraction (20KgS) (Fig. 2d). The result strongly supports the localization of catalase in microbodies, but not in the cytosol. Moreover, the type of microbody that was stained with DAB was distinct from mitochondria, as shown in Fig. 3(a, b), but it was not stained with DAB in the presence of a catalase inhibitor (data not shown) (Dijken *et al.*, 1975; Fahimi & Baumgart, 1999). Furthermore, the fact that catalase has been commonly used as a peroxisomal marker enzyme supports the peroxisomal localization of catalase in *F. palustris*. The finding is in accordance with oleate-grown *A. nidulans* (Valenciano *et al.*, 1996), but is different from glucose-grown *S. cerevisiae*, in which catalase is widely distributed in mitochondria, peroxisomes and cytosol (Petrova *et al.*, 2002).

Fig. 2. Distribution of enzyme activities in fractions of a sucrose density-gradient centrifugation (a, b and c), and differential centrifugation up to 20000 g (d). (a) Protein (■), sucrose (○); (b) FPICL1 (▲), MS (◇); (c) SDH (●), catalase (Cat; □); (d) relative enzyme activities in supernatant fraction (PNS) obtained from centrifugation at 3000 g (black bars). The activities in pellet (20 KgP; white bars) and supernatant (20 KgS; grey bars) fractions obtained from centrifugation at 20 000 g centrifugation. Data points represent means; SEM is given in (d), *n*=3.

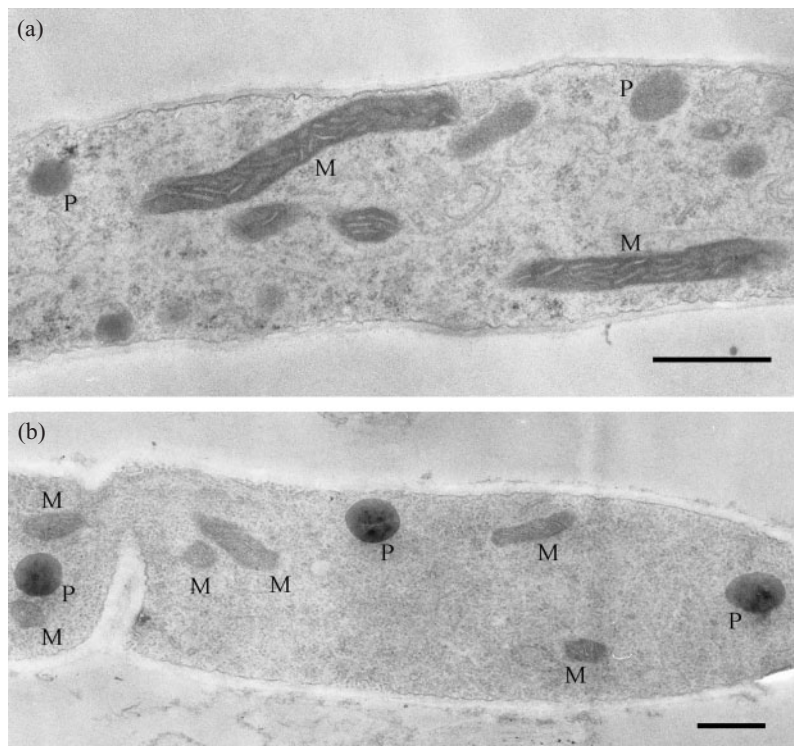


Fig. 3. Detection of catalase as a peroxisomal marker in mycelium of glucose-grown *F. palustris*. (a) Longitudinal section of a representative area of mycelium of *F. palustris*. (b) Cytochemical staining of catalase by DAB/H₂O₂. M, mitochondrion; P, peroxisome. Bars, 1 μ m.

Subcellular localization of FPICL1

FPICL1 and MS were found to be peroxisomal, because both FPICL1 and MS coexisted with catalase, but neither of them coexisted with SDH (Fig. 2a–c). Furthermore, FPICL1 and MS were not cytosolic. Greater than 60% of FPICL1, and 70% of MS, activities were recovered from the fraction containing microbody (20KgP), whereas only 10% each of

the key enzymes was from the supernatant fraction containing cytosol (20KgS) (Fig. 2d).

The peroxisomal localization of FPICL1 was further confirmed by immunocytochemical analysis with rabbit antiserum containing anti-FPICL1 antibodies. We prepared the antiserum which specifically reacted with purified FPICL1. Only one protein band corresponding to the

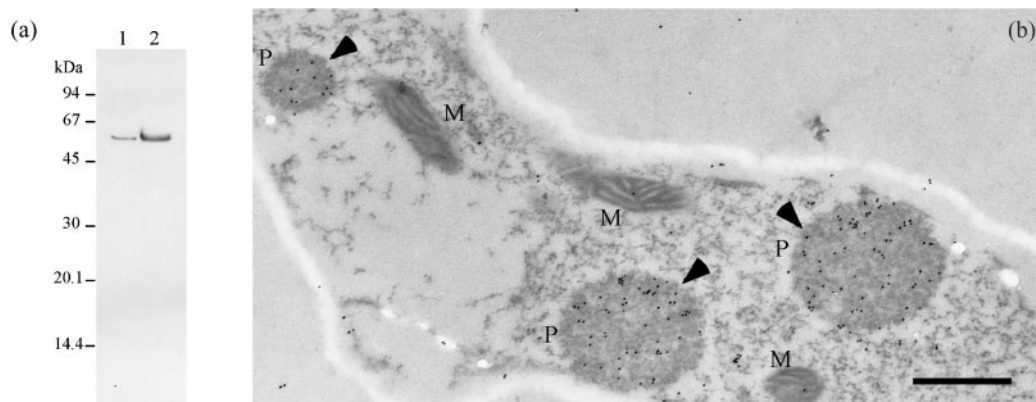


Fig. 4. Immunocytochemical localization of FPICL1 in the mycelium of glucose-grown *F. palustris*. (a) Detection of FPICL1 proteins by Western blot analysis. Proteins separated by SDS-PAGE (5 μ g in each lane) were reacted with rabbit anti-FPICL1 antibody (diluted 1:3000). Lane 1, cell-free extracts from *F. palustris*; lane 2, FPICL1 purified from the fungus. The positions of molecular mass markers are shown. (b) Immunoelectron microscopic detection of FPICL1. M, mitochondrion; P, peroxisome. Bar, 1 μ m.

enzyme appeared on the gel loaded with cell-free extracts of *F. palustris* (Fig. 4a). A significant amount of immunogold labelling was located in the peroxisomes, but not in the cytosol or mitochondria (Fig. 4b). Alternatively, peroxisomal ICLs have been reported for *Ashbya gossypii* grown on C₂ compounds (Maeting *et al.*, 1999), *C. tropicalis* (Tanaka & Ueda, 1993), *Yarrowia lipolytica* (Titorenko *et al.*, 1998), *A. nidulans* (Valenciano *et al.*, 1996), and a glyoxysome-like ICL for acetate or oleate-grown *Neurospora crassa* (Kionka & Kunau, 1985). However, to the best of our knowledge, this investigation provides the first experimental evidence for peroxisomal localization of ICL in a glucose-grown basidiomycete.

Subcellular localization of OXA and ACS

Both OXA and ACS were shown to be cytosolic. Activities of 74 % for OXA, and 66 % for ACS, were recovered from the supernatant fraction (20Kgs), whereas activities of less than

10 % for each enzyme were recovered from the pellet fraction (20Kgp) (Fig. 2d). We found that there was no difference in MDH activity between the cytosolic and the pellet fractions (data not shown), which suggested that MDH isozymes were distributed in various organelles and cytosol in a cell of *F. palustris*. A similar finding has been reported for *S. cerevisiae*, in which MDH isozymes are distributed throughout the mitochondria, peroxisomes and cytosol (Steffan & McAlister-Henn, 1992).

Features of carbon metabolism of *F. palustris*

Although we previously proposed that ICL was shared by the TCA and GLOX cycles because of isocitrate metabolism (Munir *et al.*, 2001b), we propose a revised model of carbon metabolism in relation to oxalate biosynthesis, as shown by the enzyme localizations in Fig. 5. The TCA and GLOX cycles, and acetate recycling, have been proposed to be involved constitutively in carbon metabolism for oxalate

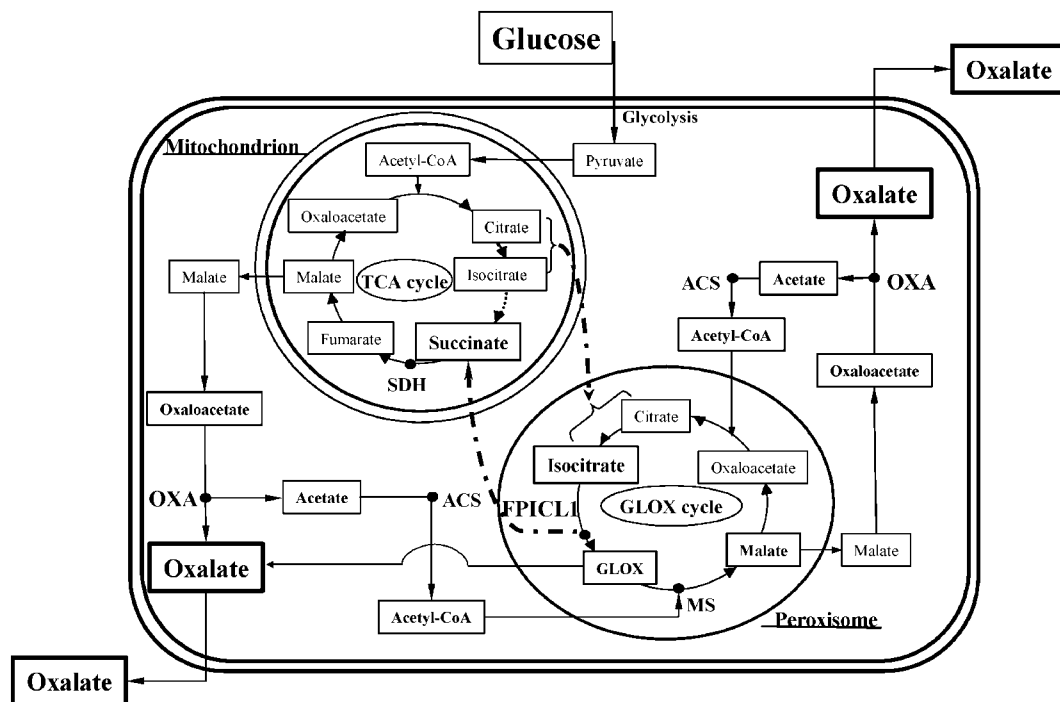


Fig. 5. Possible mechanisms for carbon metabolism in relation to oxalate biosynthesis in *F. palustris*, considering the localization of the TCA and GLOX cycles, and acetate recycling. Each metabolic step involved in the TCA and GLOX cycles, and acetate recycling, shown as normal arrows, has been proposed on the basis of enzyme activities that we detected from the mycelia of *F. palustris* (Munir *et al.*, 2001b). However, we have not investigated the enzymes involved in the conversion of glucose to acetyl-CoA via pyruvate. Localization of the TCA and GLOX cycles, and acetate recycling, are proposed based on SDH, and FPICL1 and MS for the TCA and GLOX cycles, respectively, and ACS and OXA for acetate recycling. However, the localization of enzymes not shown in Fig. 5 remains to be elucidated. Transportation of malate to the cytosol has not been investigated for *F. palustris*, although the transportation is proposed to occur. Also, we have not shown experimental evidence for the transportation of succinate, citrate or isocitrate, and acetyl-CoA. Furthermore, the mechanism for oxalate excretion has not been elucidated. The dotted arrow indicates a small amount of conversion of isocitrate to succinate, since ODH activity has not been detected, and there is less activity of IDH than of FPICL1 (Munir *et al.*, 2001b). The dot-and-dash arrow indicates speculative transportation between mitochondria and peroxisomes.

fermentation of glucose-grown *F. palustris*, on the basis of enzyme activities detected from the mycelia catalysing each metabolic step shown in Fig. 5 (Munir *et al.*, 2001b). The findings of peroxisomal FPICL1 and MS suggest a peroxisomal GLOX cycle, although the peroxisomal localization of MDH, citrate synthase and aconitase needs to be demonstrated for conclusive confirmation of a peroxisomal GLOX cycle. On the other hand, the presence of mitochondrial SDH suggests a mitochondrial TCA cycle, although the mitochondrial localization of fumarase, MDH, citrate synthase and aconitase remains to be investigated in order to confirm this. In this context, it is noteworthy that conversion of the C₆ carboxylic acid to succinate through the TCA cycle is not thought to be significant, because a lower level of IDH activity than FPICL1 activity, and a lack of ODH activity, have been detected from the mycelia of *F. palustris* (Munir *et al.*, 2001b). The presence of cytosolic ACS and OXA suggests that acetate recovery (Munir *et al.*, 2001b) occurs in the cytosol, although the presence of cytosolic MDH needs to be demonstrated in order to confirm a cytosolic acetate recovery process. On the other hand, we have not investigated enzymes involved in conversion of glucose to acetyl-CoA via pyruvate.

Accordingly, we found that the FPICL1 in the GLOX cycle was not shared with the TCA cycle in mitochondria. It is proposed that the peroxisomal GLOX cycle, the mitochondrial TCA cycle, and cytosolic acetate recovery function in coordination with oxalate fermentation in *F. palustris*. The results allow us to postulate the following metabolite transportations for glucose-grown *F. palustris*, although we have not yet proved them. (1) Succinate may be transported from peroxisomes to mitochondria to replenish metabolite carbons for the TCA cycle. This hypothesis is supported by the separate localization of peroxisomal FPICL1 and mitochondrial SDH, together with the absence of ODH activity (Munir *et al.*, 2001b). This speculated transportation of succinate is different from the inducible succinate transportation from the cytosol to mitochondria in *S. cerevisiae* grown on a non-fermentable carbon source (Flores *et al.*, 2000). (2) Isocitrate or citrate transportation from mitochondria to peroxisomes may occur to solve the problem caused by the lesser catabolic activity for C₆ carboxylic acid metabolism in the TCA cycle than in the GLOX cycle (Munir *et al.*, 2001b). (3) Acetyl-CoA formed in the cytosol must be transported to mitochondria to be catabolized because of the large amount of acetate produced concomitantly with oxalate (Munir *et al.*, 2001b). A similar process of acetyl-CoA formation and utilization, concomitant with oxalate production, has been proposed for *A. niger* (Ruijter *et al.*, 1999). Alternatively, acetyl-CoA must be transported to peroxisomes as a substrate for MS and citrate synthase. However, the dual localization of citrate synthase and aconitase in mitochondria and peroxisomes remains to be elucidated, although dual localization of citrate synthase occurs in *S. cerevisiae* (Lee *et al.*, 2000). These transportations are postulated to occur constitutively, because oxalate fermentation is indispensable for the growth of *F. palustris*

(Munir *et al.*, 2001b). However, further research is needed to elucidate the mechanisms of transportation of the organic acid metabolites within a cell of *F. palustris*.

In conclusion, FPICL1 and MS are key enzymes of the GLOX cycle, and occur constitutively in peroxisomes in *F. palustris* grown on glucose. An acetate-recovery system operates in the cytosol, and thus organic acid metabolites involved in oxalate biosynthesis are finally oxidized to oxalate.

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