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Characterization of *Aspergillus nidulans* peroxisomes by immunoelectron microscopy

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Abstract In previous work, we have demonstrated that oleate induces a massive proliferation of microbodies (peroxisomes) in *Aspergillus nidulans*. Although at a lower level, proliferation of peroxisomes also occurs in cells growing under conditions that induce penicillin biosynthesis. Here, microbodies in oleate-grown *A. nidulans* cells were characterized by using several antibodies that recognize peroxisomal enzymes and peroxins in a broad spectrum of eukaryotic organisms such as yeast, and plant, and mammalian cells. Peroxisomes were immunolabeled by anti-SKL and anti-thiolase antibodies, which suggests that *A. nidulans* conserves both PTS1 and PTS2 import mechanisms. Isocitrate lyase and malate synthase, the two key enzymes of the glyoxylate cycle, were also localized in these organelles. In contrast to reports of *Neurospora crassa*, our results demonstrate that *A. nidulans* contains only one type of microbody (peroxisomes) that carry out the glyoxylate cycle and contain 3-ketoacyl-CoA thiolase and proteins with the C-terminal SKL tripeptide.

Key words *Aspergillus nidulans* · Peroxisomes · Peroxisome biogenesis · PTS1 · PTS2 · Glyoxylate cycle · Microbodies · Isocitrate lyase · Malate synthase

Introduction

The filamentous fungus *Aspergillus nidulans* is able to grow on fatty acids (such as oleate) or C₂ compounds

(such as acetate) as the sole carbon source. Growth on oleate requires the induction of fatty acid β -oxidation and glyoxylate cycle enzymes since mutants defective in isocitrate lyase or malate synthase activities are unable to grow on this carbon source (De Lucas et al. 1997b).

Recently, we have shown that the addition of oleate to the medium induces a massive proliferation of microbodies (peroxisomes) in *A. nidulans* (Valenciano et al. 1996). This proliferation of peroxisomes is accompanied by the induction of several enzymes including the fatty acid β -oxidation enzymes and isocitrate lyase. These enzymes were localized in an organellar fraction that was clearly separated from mitochondria by isopycnic centrifugation (Valenciano et al. 1996). These results suggested a peroxisomal location of these enzymes. Nevertheless, complementary studies were necessary to confirm the peroxisomal localization of the fatty acid β -oxidation pathway and glyoxylate cycle enzymes in *A. nidulans*. These studies would also revealed interesting aspects of the function and biogenesis of peroxisomes in filamentous fungi.

The biogenesis of peroxisomes involves new synthesis of peroxisomal proteins on free polyribosomes in the cytoplasm and their translocation into the peroxisomal membrane or matrix (Subramani 1993). Proteins destined for the peroxisome matrix contain specific, conserved molecular determinants that target them to the organelle and are termed PTS1 and PTS2. The PTS1 signal implies the carboxy-terminal –SKL–COOH and related motifs (Gould et al. 1988, 1989) and has been shown to be able to direct most matrix proteins to peroxisomes (Subramani 1996). The second PTS signal (PTS2) was firstly discovered at the amino-terminal 11 amino acids of rat peroxisomal 3-ketoacyl-CoA thiolase as necessary for import of this enzyme into the peroxisomes (Osumi et al. 1991, Swinkels et al. 1991). Mutational and comparative analyses have revealed that the conserved N-terminal nonapeptide for the PTS2 sequence is (R/K)(L/V/I)X5(H/Q)(L/A) (Rachubinski and Subramani 1995, Subramani 1996). The PTS2 sequence is highly conserved among human, rat, and several fungal thiolases.

In comparison with yeasts, where the function of peroxisomes and their biogenesis are well-known processes

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[for recent reviews, see Elgersma and Tabak (1996) and Waterham and Cregg (1997)], only a very few studies of filamentous fungi have analyzed the role of peroxisomes. Nevertheless, these reports have stressed the importance of microbodies (peroxisomes) in specific processes of these microorganisms, e.g., the penicillin biosynthesis of *Penicillium chrysogenum* and the karyogamy of *Podospora anserina* (Berteaux-Lecellier et al. 1995). Müller et al. (1991a) have localized the final enzyme involved in penicillin biosynthesis, acyl CoA:isopenicillin *N*-acyltransferase (acyltransferase), in microbodies of *P. chrysogenum*. It has also been shown that the conserved PTS1 signal (–ARL–COOH) at the C-terminus of *P. chrysogenum* acyltransferase is essential for targeting the enzyme to peroxisomes (Müller et al. 1992).

A. nidulans is a very attractive species for the study of the biogenesis of peroxisomes in filamentous fungi. Since *A. nidulans* is able to produce penicillin and, unlike *P. chrysogenum*, is amenable to classic and molecular genetic analyses, studies on peroxisome biogenesis in this fungus should help to understand the involvement of these organelles in several metabolic pathways, including penicillin production.

This work represents the first characterization of peroxisomes in a filamentous fungus by immunocytochemistry. Our results show that *A. nidulans* peroxisomes carry out the glyoxylate cycle and contain the last enzyme of the fatty acid β -oxidation pathway as well as proteins containing the PTS1 peroxisomal targeting signal.

Materials and methods

Strain and growth conditions

A. nidulans R21 (*paba A1 yA2*) (Armitt et al. 1976) was grown in *Aspergillus* minimal medium (Pontecorvo et al. 1953) supplemented with the auxotrophic requirement (1 μ g *p*-aminobenzoic acid ml⁻¹). Cultures [200 ml of 2% (w/v) glucose minimal medium in 500-ml Erlenmeyer flasks] were inoculated with 4 ml of suspension (2 \times 10⁸ conidia ml⁻¹) and incubated at 37°C on a rotary shaker at 200 rpm for 18 h. At this stage, the mycelium was harvested by filtration through muslin, washed twice with 50 mM sodium phosphate buffer (pH 7.0), and divided into two aliquots. Each aliquot was transferred to a 250-ml Erlenmeyer flask containing 100 ml of *Aspergillus* minimal medium, 1 μ g *p*-aminobenzoic acid ml⁻¹ and 6 mM oleate in 1% Tergitol NP-40 (De Lucas et al. 1997b) as sole carbon source. Incubation continued under the same conditions for 12 h. At this time, the mycelia were harvested and washed as described before. Samples of these mycelia were processed for electron microscopy techniques. The remaining mycelia were frozen at –70°C

To analyze the ultrastructure of *A. nidulans* R21 cells producing penicillin V, 1 ml of suspension (2 \times 10⁸ conidia ml⁻¹) was inoculated in 250-ml Erlenmeyer flasks containing 20 ml of seed medium (Brakhage et al. 1992) plus 1 μ g *p*-aminobenzoic acid ml⁻¹. The cultures were incubated on a rotary shaker (250 rpm) at 26°C for 24 h. The mycelia were harvested, washed with 0.8% (w/v) NaCl and transferred to 250-ml Erlenmeyer flasks containing 20 ml of fermentation medium (Brakhage et al. 1992) plus 1 μ g *p*-aminobenzoic acid ml⁻¹. This fermentation medium contains corn steep solids (Sigma), a major component of most penicillin production media, and phenoxyacetic acid (Sigma), which acts as a precursor of penicillin V (Brakhage and Turner 1995). Incubation continued under the same conditions for 48 h. Fresh mycelium

was used for electron microscopy methods. The remaining mycelium was stored at –70°C

SDS Gel Electrophoresis and Western Blotting

Crude extracts were prepared as described before (De Lucas et al. 1994). Protein concentration was determined according to Bradford (1976) using the BioRad Protein Assay Kit (BioRad, Munich, Germany) and bovine serum albumin (BSA) as standard. Equal amounts of protein (40 μ g) were separated by the method of Laemmli (1970) on 10% polyacrylamide slab gels containing 1% SDS. Low-molecular-mass (LMW) standards (Biolabs, New England) were employed to estimate the molecular weight of protein bands. Proteins were transferred onto nitrocellulose after SDS-PAGE using a semi-dry electroblotter according to Kynhse-Anderse (1984).

Antibodies

Polyclonal antibodies against 3-ketoacyl-CoA-thiolase from *Saccharomyces cerevisiae* (Erdmann and Kunau 1993), malate-synthase from *Candida tropicalis* (McCammon et al. 1990), acyl-CoA:isopenicillin *N*-acyltransferase from *Penicillium chrysogenum* (Müller et al. 1991a), and anti-SKL antibodies (Gould et al. 1990) were used in this work. These antisera were provided by M. Veenhuis (University of Groningen, Groningen, The Netherlands). Polyclonal antibodies raised against purified *A. nidulans* isocitrate lyase were also utilized (De Lucas et al. 1997a).

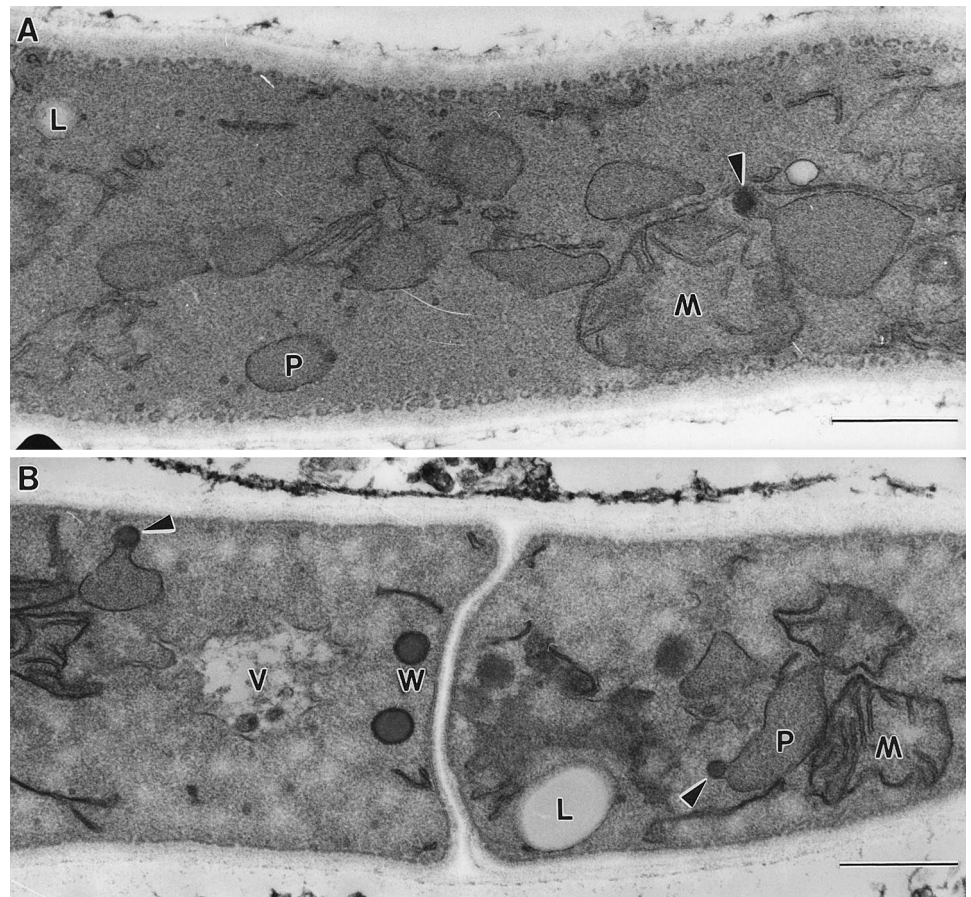
For detection of the respective proteins on Western blots, the primary antibodies were diluted 1:10,000 in TBST buffer [30 mM NaCl, 0.01% Tween-20, and 2 mM Tris-HCl (pH 8)]. Alkaline-phosphatase-conjugated goat anti-rabbit IgG (Boehringer, Mannheim, Germany) was used as a secondary antibody at the same dilution. The immune complexes were visualized by adding 200 μ l 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Boehringer) to 10 ml of a solution containing 5 mM MgCl₂, 0.1 M NaCl, and 0.1 M Tris-HCl (pH 9.5).

Electron microscopy and immunocytochemistry

Mycelia grown in oleate minimal medium or fermentation medium were fixed in 1.5% (w/v) KMnO₄ for 20 min at room temperature. Samples were stained in 1% (w/v) aqueous uranyl acetate at room temperature for 1–3 h. After dehydration in a graded ethanol series at room temperature, the samples were embedded in Epon 812 (Luft 1961) at 100°C for 4 h. Ultra-thin sections were cut with a diamond knife and examined in a Phillips EM 201 transmission electron microscope operating at 60 kV. The volume density of peroxisomes in the cytoplasm of cells growing on oleate-containing or fermentation medium was estimated by the point-counting technique according to Weibel and Bolender (1973).

For immunocytochemistry, intact cells were fixed in 2.5% (v/v) formaldehyde and 0.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 0°C, dehydrated in a graded ethanol series at room temperature, and embedded in Unicryl (Electron Microscopy Sciences, USA) following the manufacturer's instructions. After polymerization of the resin, ultra-thin sections were cut with a diamond knife and mounted on Formvar carbon nickel grids. The sections were blocked with PBSG buffer [8 mM Na₂HPO₄, 1 mM NaH₂PO₄, 135 mM NaCl, 2 mM KCl, and 20 mM glycine (pH 9.0)] containing 0.5% (w/v) BSA and incubated with the primary antibodies (1:1,000 in blocking solution) overnight at 4°C. After three washes in PBSG buffer, sections were incubated in goat anti-rabbit IgG conjugated with 15 nm gold particles (Amersham Life Science, USA) diluted 1:20 in blocking buffer for 1 h at room temperature. Sections were washed in PBSG buffer and distilled water and were stained with 1% (w/v) aqueous uranyl acetate containing 0.1% (w/v) methylcellulose.

Fig. 1 A, B Electron micrographs of hyphae of *Aspergillus nidulans*. **A** Longitudinal section of a representative area of hyphae of *A. nidulans* grown on oleate minimal medium showing the oleate-induced proliferation of peroxisomes. **B** Section of a typical hypha of *A. nidulans* growing on medium that induces penicillin production. Peroxisomes showed round to elongate shape and made up to 1.5% of the cytoplasmic volume. Note the development of Woronin bodies from peroxisomes (arrowheads in **A** and **B**) in hyphae of *A. nidulans* fixed with KMnO_4 . Bars represent 0.5 μm ; L lipid body, P peroxisome, M mitochondrion, V vacuole, and W Woronin body)



Results and discussion

Ultrastructure of *A. nidulans* cells grown in oleate-containing and penicillin production media.

Recently, we have described the oleate-induced proliferation of microbodies (peroxisomes) in *A. nidulans* (Valenciano et al. 1996). Peroxisomes of oleate-grown cells appeared in clusters, were rounded to elongated in shape, measured up to a maximum 1.5 μm in length, and made up to 6% of the cytoplasmic volume (Valenciano et al. 1996). In addition to these peroxisomes, other small organelles characteristic of certain ascomycetes, the so-called Woronin bodies, were observed in the proximity of septa. Both peroxisomes and Woronin bodies were characterized by the presence of the enzyme catalase, as shown by the diaminobenzidine (DAB)-specific staining (Valenciano et al. 1996).

In *Fusarium oxysporum*, Wergin (1973) has shown that the Woronin bodies are produced by microbodies. Development of the Woronin body from a microbody includes the formation of a single paracrystalline inclusion that is gradually extruded and separated from the parent organelle by an exocytic mechanism. In previous ultrastructural analyses of oleate-grown cells of *A. nidulans* fixed with glutaraldehyde and post-fixed with OsO_4 , we could

not clearly see the formation of Woronin bodies (Valenciano et al. 1996). Nevertheless, when hyphae of *A. nidulans* were fixed with KMnO_4 , the development of Woronin bodies from peroxisomes was clearly observed (Fig. 1 a,b). It has been reported that chemical fixation of cells (e.g., permanganate fixation) causes structural alterations, and dehydration at room temperature introduces shrinkage of the overall structure of the cell (Müller et al. 1991 b); therefore, it will be of interest to study the development of Woronin bodies from peroxisomes in *A. nidulans* by a better-preserving technique such as freeze-substitution. The proposed role of the Woronin bodies is to regulate the cytoplasmic flow between adjacent cells. Moreover, when a cell is injured, the Woronin bodies initially fuse and plug the septal pore with their homogeneous content to avoid the loss of cytoplasm (Wergin 1973).

Müller et al. (1991 a) have shown that there is a correlation between the proliferation rate of microbodies and the capacity for penicillin production in different strains of *P. chrysogenum*. *A. nidulans*, like *P. chrysogenum*, produces penicillin and contains the enzyme acyltransferase, for which a peroxisomal localization has been suggested (Brakhage and Turner 1995). Since the microbodies could play a role in penicillin biosynthesis of *A. nidulans*, we studied the ultrastructure of wild-type cells grown under penicillin-inducing conditions to determine the proliferation rate of peroxisomes.

Mycelia grown for 48 h in the fermentation medium and producing approximately 75% of the maximal levels of penicillin V (De Lucas et al. 1997b) were fixed in KMnO_4 and observed by electron microscopy. In comparison with oleate-grown cells (Fig. 1 a; Valenciano et al. 1996), relatively few peroxisomes were detected (Fig. 1 b). These organelles were round in shape, measured up to a maximal length of 0.7 μm , and made up 1.5% of the cytoplasmic volume. The size and volume fraction of peroxisomes of *A. nidulans* cells growing on fermentation medium were greater than the size (0.2 μm) and volume fraction (0.26%) of peroxisomes of glucose-grown cells (repressing conditions) (Valenciano et al. 1996; De Lucas et al. 1997 b), demonstrating that proliferation of peroxisomes occurs in penicillin-producing cells. The level of peroxisome proliferation under these growth conditions resembled that observed in *A. nidulans* cells grown on acetate (Valenciano et al. 1996), a C_2 compound that supposedly requires microbodies in order to be metabolized.

Detection of putative peroxisomal proteins of *A. nidulans* by Western blotting

Recently, we localized the fatty acid β -oxidation enzymes in an organellar fraction that was clearly separated by isopycnic centrifugation from the mitochondrial peak fraction (Valenciano et al. 1996). These results suggested a peroxisomal localization of these enzymes in the filamentous fungus *A. nidulans*. In order to confirm this hypothesis and extend our knowledge of the function and biogenesis of peroxisomes in *A. nidulans*, we planned to investigate the cellular localization of 3-ketoacyl-CoA thiolase and proteins containing an SKL targeting signal. In addition, we also studied the localization of the two key enzymes of the glyoxylate cycle, namely malate synthase and isocitrate lyase. For this purpose, we used various available polyclonal antibodies that recognize the respective proteins in yeast, plant, or mammalian cells (Gould et al. 1990; Mc Cammon et al. 1990; Liu et al. 1995). In addition, we used antibodies raised against isocitrate lyase from *A. nidulans* (De Lucas et al. 1997 a).

Proteins from crude extracts of cells grown in oleic acid medium were subjected to SDS-PAGE, and the proteins were blotted on to nitrocellulose membranes. In Western blots labeled with *S. cerevisiae* anti-thiolase antibodies, a single band of approximately 45 kDa was detectable (Fig. 2). The molecular mass of this band is similar to that reported for the 3-ketoacyl CoA thiolase monomer in several yeasts including *C. tropicalis* (Kurihara et al. 1989), *S. cerevisiae* (Erdman and Kunau 1994), and *Yarrowia lipolytica* (Berninger et al. 1993). Since this band was absent in noninduced glucose-grown cells, we anticipated that the 45-kDa band recognized by the anti-thiolase serum indeed represents the *A. nidulans* 3-ketoacyl CoA thiolase.

C. tropicalis malate synthase antiserum recognized a major band of 60 kDa. A band of identical mass (60 kDa) was also detected in blots probed with anti-SKL antibod-

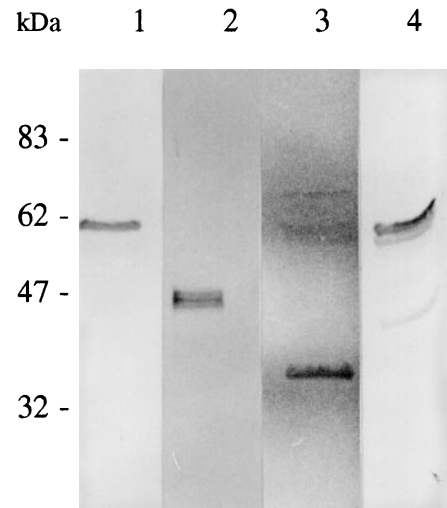


Fig. 2 Detection of putative peroxisomal proteins by Western blotting. Proteins from crude extracts of *Aspergillus nidulans* oleate-grown cells were separated on an SDS-10% polyacrylamide gel, blotted onto nitrocellulose membranes and colored with specific antibodies against 1 isocitrate lyase, 2 3-ketoacyl-CoA thiolase, 3 carboxy-terminal SKL-COOH sequence, and 4 malate synthase. The position of molecular mass markers (LMW Biolabs, New England) is shown

ies (Fig. 2). The *A. nidulans* malate synthase contains a C-terminal SKL sequence and a mol. mass of 60 kDa (Sandeman et al. 1991). Thus, it is very likely that the major band at 60 kDa detected in Western blotting by the anti-malate synthase serum corresponds to the *A. nidulans* malate synthase. The minor band of 43 kDa recognized by the malate synthase antiserum might represent a stable degradation product of the enzyme (Fig. 2).

Antibodies against *A. nidulans* isocitrate lyase detected a band whose molecular mass corresponded to that of the monomer (59 kDa) (De Lucas et al. 1997 a). The anti-SKL antibodies that have been reported to be specific for the C-terminal SKL-COOH sequence (Gould et al. 1990; Keller et al. 1991) recognized, apart from the 60-kDa band, at least three different bands of approximately 68, 58, and 37 kDa. (Fig. 2). These proteins have not yet been identified.

Detection of peroxisomes and peroxisomal enzymes in *A. nidulans* by immunoelectron microscopy

The term "microbodies" refers only to a morphological entity and implies no specific function. The microbodies have been termed glyoxysomes or peroxisomes depending on their enzyme content and metabolic function. Microbodies housing the glyoxylate cycle and fatty acid β -oxidation enzymes have been called glyoxysomes (Trelease et al. 1971; Keller et al. 1991), while those containing catalase and hydrogen-peroxide-generating oxidases and performing the fatty acid β -oxidation have been named peroxisomes. In plants (Trelease et al. 1971; De Bellis et al. 1990; Keller et al. 1991), glyoxysomes may convert to peroxisomes (and vice versa) by the gradual re-

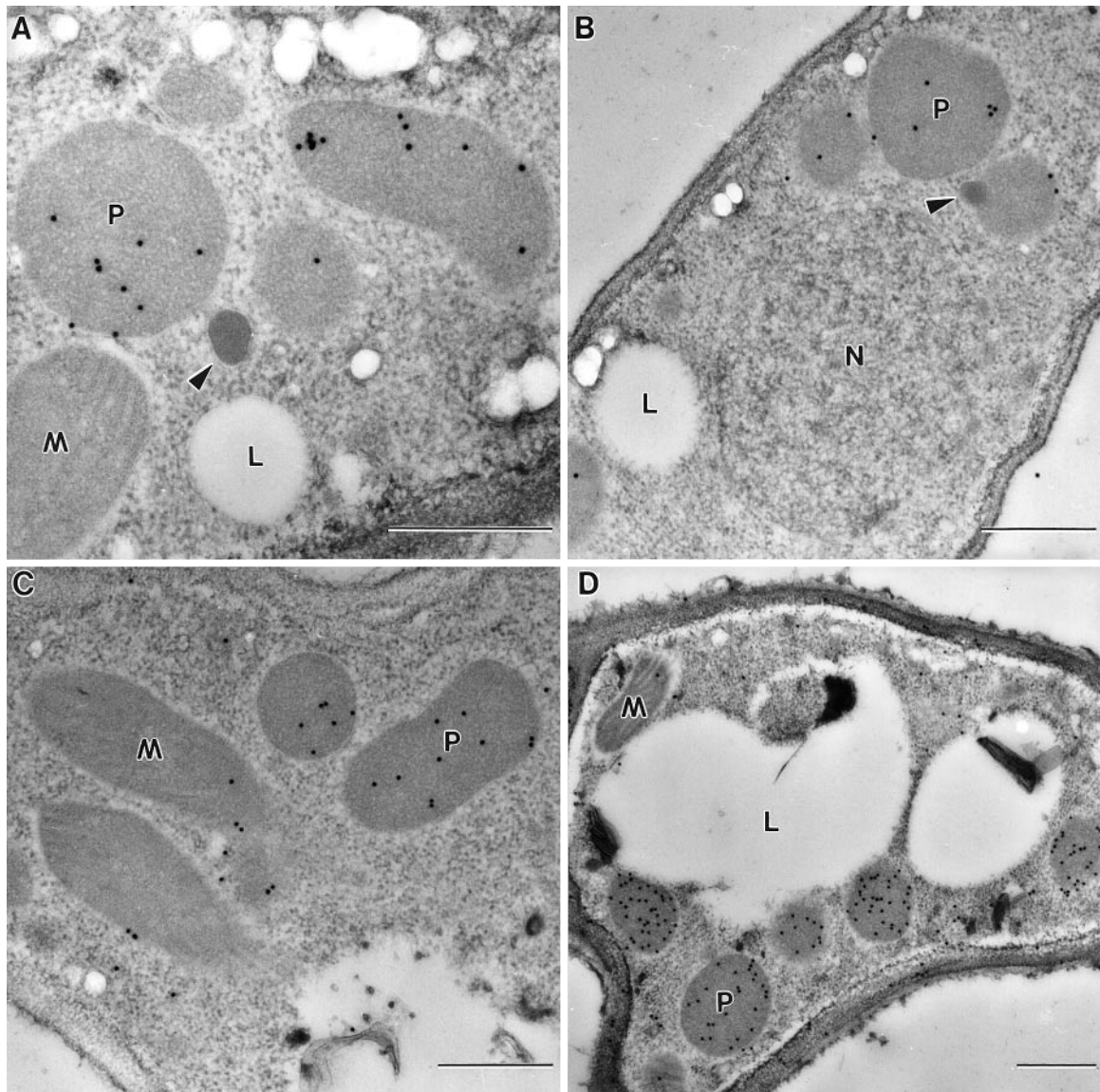


Fig. 3 Immunocytochemical localization of 3-ketoacyl-CoA thiolase (**A**), proteins containing the C-terminal SKL tripeptide (**B**), malate synthase (**C**), and isocitrate lyase (**D**) in *A. nidulans* oleate-grown cells. Antibodies against *Saccharomyces cerevisiae* 3-ketoacyl-CoA thiolase, SKL tripeptide at the COOH terminus, *Candida tropicalis* malate synthase, and *A. nidulans* isocitrate lyase were used. As can be clearly seen, location of the enzymes was restricted to the peroxisome matrix. In addition, formation of Woronin bodies from peroxisomes is also shown (arrowhead in **A** and **B**). (bars 0.5 μm ; P peroxisome, L lipid body, M mitochondrion, and N nucleus)

placement of their enzyme content. However, in the ascomycete *Neurospora crassa*, a species closely related to *A. nidulans*, glyoxysomes and peroxisomes appear to be different organelles that coexist within the same cell at the same time (Wanner and Theimer 1982; Kionka and Kunau 1985; Keller et al. 1991). We should be able to determine immunocytochemically, by using the specific antibodies mentioned above, whether one or two types of microbodies exist in *A. nidulans* cells.

The results, summarized in Fig. 3, demonstrate that the *S. cerevisiae* anti-thiolase serum specifically labeled the *A. nidulans* peroxisomes (Fig. 3a). All other peroxisomal thiolases known to date contain a PTS2 sequence that sorts the protein to the peroxisomal matrix (Swinkels et al. 1991; Subramani 1993; Waterham and Cregg 1997). We speculate that *A. nidulans* 3-ketoacyl-CoA thiolase contains a PTS2 sequence, indicating that this filamentous fungus conserves a PTS2 import machinery. However, further studies to demonstrate that *A. nidulans* 3-ketoacyl CoA thiolase indeed contains a PTS2 import signal remain to be done.

Using anti-SKL antibodies, the same type of organelles (peroxisomes) that were detected with the anti-thiolase serum were again labeled (Fig. 3b). Similar results were obtained using antibodies against isocitrate lyase and malate synthase (Fig. 3c and d). These results – all four antisera tested labeled all microbody profiles present in the sections – clearly indicate that the corresponding proteins were located in one and the same organelle and,

hence, that only one type of microbody (peroxisomes) exists in *A. nidulans*. This result was in line with our previous work, which revealed that all microbodies (peroxisomes) observed in *A. nidulans* cells by electron microscopy contained the peroxisomal enzyme catalase (Valenciano et al. 1996).

In *S. cerevisiae*, it has been described that the glyoxylate cycle may take place in a dual localization (peroxisomes and cytoplasm; McCammon et al. 1990) since malate synthase is a peroxisomal enzyme but isocitrate lyase appears to be cytosolic. Immunolocalization of malate synthase and isocitrate lyase reported in this work clearly shows the compartmentalization of the glyoxylate cycle in peroxisomes of *A. nidulans*.

Analysis of amino acid sequence of *A. nidulans* isocitrate lyase (Gainey et al. 1992) shows the absence of putative PTS1 or PTS2 targeting signals for sorting the protein into peroxisomes. Besides the PTS1 and PTS2 signals, additional internal motifs have been reported as being involved in sorting a few matrix enzymes to peroxisomes (Small et al. 1988; Kragler et al. 1993; Elgersma et al. 1995). Kamasawa et al. (1996) have recently shown that an internal region of the *C. tropicalis* isocitrate lyase is responsible for targeting the heterologous protein to *S. cerevisiae* peroxisomes. It would be interesting, therefore, to investigate whether alternative targeting signals also exist for isocitrate lyase of *A. nidulans*.

Woronin bodies were also observed in the numerous sections subjected to the immunogold procedure (Fig. 3a), but they were never labeled by the antisera used. This suggests that although Woronin bodies may derive from microbodies (peroxisomes), they present a different matrix content.

Finally, our attempts to localize in *A. nidulans* the acyl CoA:isopenicillin *N*-acyltransferase using available anti-acyltransferase antibodies against the *P. chrysogenum* enzyme were unsuccessful due to a very low level of cross-reactivity. Further studies involving several techniques of molecular biology to localize *A. nidulans* acyltransferase are now in progress.

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