Immunoelectron Microscopic Localization of Thiolases, β -Oxidation Enzymes of an *n*-Alkane-Utilizable Yeast, *Candida tropicalis*

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ABSTRACT. The location of acetoacetyl-CoA thiolase (T-I) and 3-ketoacyl-CoA thiolase (T-III), enzymes of the fatty acid β -oxidation system, was studied in *n*-alkane-grown *Candida tropicalis* cells by immunoelectron microscopy using a post-embedding method with colloidal gold conjugated IgG. The deposition of gold particles for T-I was detected in the microbodies and cytoplasm and that of gold particles for T-III specifically in the microbodies. The double labeling technique confirmed that T-I and T-III occurred concurrently in a microbody and T-I also in cytoplasm. These results were consistent with the biochemical data based on subcellular fractionation and indicated that the yeast β -oxidation system operates efficiently only in the microbodies.

A large number of microbodies were developed in the cells of *Candida tropicalis* grown in *n*-alkanes as a sole carbon source (4, 5). Enzymes of the fatty acid β -oxidation system were inducibly synthesized in parallel with the profuse appearance of microbodies.

In mammalian cells, long chain fatty acids are assumed to be degraded in the microbodies, from which process medium or short chain fatty acids are subsequently transported from microbodies to mitochondria to be degraded to acetyl-CoA, the substrate of the tricarboxylic acid cycle. In the *n*-alkane-grown *Candida tropicalis* cells, however, the enzyme activity of the fatty acid β -oxidation system is detected only in the microbodies (1). Kurihara *et al.* (2, 3) purified and characterized two types of thiolases, acetoacetyl-CoA thiolase (T-I) and 3-ketoacyl-CoA thiolase (T-III), from the microbodies of *n*-alkane-grown *Candida tropicalis* cells. T-III is responsible for the degradation of long chain acyl-CoAs and T-I is specific to acetoacetyl-CoA. These findings suggested that the complete degradation of fatty acids is carried out in yeast microbodies through the cooperation of these two thiolases. As these reactions are the final step in the fatty acid β -oxidation system, the determination of their precise location is important for the evaluation of the function of this system.

Abbreviations: T-I, acetoacetyl-CoA thiolase; T-III, 3-ketoacyl-CoA thiolase.

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We describe here immunoelectron microscopic confirmation of the location of these two types of thiolases, T-I and T-III.

MATERIALS AND METHODS

Strain and growth. *Candida tropicalis* pK233 (ATCC 20336) was grown to mid-logarithmic phase in a medium containing an *n*-alkane mixture (C₁₀-C₁₃) as described elsewhere (7).

Conventional electron microscopy. Yeast cells were fixed with 2.5% glutaraldehyde. Fixed cells were treated with zymolyase 20T for digestion of cell wall, postfixed with 4% osmium tetroxide, and embedded in Quetol 812 resin as described elsewhere (5).

Immunoelectron microscopy. The fixation and embedding of the yeast cells was done using a modification (Naito *et al.*, J. Electron Microsc. 40: 242, 1991) of the procedure of Tuinen and Riezman (6). Yeast cells were fixed with a mixture of 0.5% glutaraldehyde and 3% paraformaldehyde in PBS (0.1M phosphate buffer containing 0.8% NaCl, pH 7.6) for 2 hr at room temperature. After washings three times with PBS, the cells were treated with 1% sodium metaperiodate for 15 min and 50 mM ammonium chloride for 30 min, washed again with PBS and placed into 2% low-melt agarose. The specimens were dehydrated through a graded ethylene glycol series (50 to 95%, v/v), embedded in Lowicryl K4M resin and polymerized under ultraviolet irradiation at -20°C. Ultra-

Fig. 1-2.

thin sections of the cells were cut and placed on a nickel grid 300 mesh.

Immunostaining of ultrathin sections. The grids were first incubated on a drop of 1.5 mg/ml normal goat IgG in TBS (50 mM Tris-HCl buffer containing 0.8% NaCl, pH 7.6) for 30 min at room temperature, then placed directly on antiserum against T-I or T-III in 0.1% BSA (fraction V, Sigma Chem. Co.)/TBS (1 : 1000 or 1 : 2000 dilution) for 2 hr at room temperature. For negative control, the grids were incubated with non-immune rabbit IgG (JACKSON IMMUNORESEARCH LABS, INC.) in 0.1% BSA/TBS. After incubation, the grids were washed with 0.1% BSA/TBS by sequential transfer through three changes. They were then placed on a drop of 10 nm or 20 nm colloidal gold goat anti-rabbit IgG conjugate (BIOCELL RESEARCH LAB.) (1 : 40 dilution with 0.1% BSA/TBS) for 30 min at room temperature and washed with 0.1% BSA/TBS and distilled water. Some grids were double-labeled with two different sizes of colloidal gold IgG conjugate. One face of the grids was incubated with anti-T-I antiserum followed by large particles (20 nm) of colloidal gold and the other face with anti-T-III antiserum followed by small particles (10 nm). The specimens were stained with 6% uranyl acetate, coated with carbon and examined with a JEM 1200EXS electron microscope at 120KV.

Antisera. The antisera against purified T-I and T-III used in this study were prepared as described previously (3). Absence of the cross-reaction between these antisera was demonstrated by the Western blotting method (9).

Subcellular fractionation. For subcellular fractionation, the cell wall was lysed with Zymolyase 20T and the protoplasts thus prepared were homogenized with a Teflon homogenizer. The homogenate was fractionated by differential centrifugation as described elsewhere (8). The fractions obtained were: P₂ fraction (20,000 × g pellet), microbodies and mitochondria; S₂ fraction (20,000 × g supernatant), cytoplasm and microsomes.

Enzyme assay. T-I was assayed using acetoacetyl-CoA (for acetoacetyl-CoA thiolase activity) and T-III using 3-keto-octanoyl-CoA (for 3-ketoacyl-CoA thiolase activity) (2,3).

Chemicals. 3-keto-octanoyl-CoA was prepared enzymatically from octenoyl-CoA, synthesized by the mixed anhydride method as described previously (2,3).

RESULTS AND DISCUSSION

Figure 1 shows a typical electron micrograph of an *n*-alkane-grown cell of *Candida tropicalis* pK233 in mid-logarithmic phase. A profusion of microbodies (Mb) surrounded by a single unit membrane is observed and the other organelles, nucleus (N), vacuole (V), mitochondria (M) can be seen.

Fig. 1. Ultrastructure of *Candida tropicalis* pK233 grown on *n*-alkane. N: nucleus, V: vacuole, M: mitochondrion, Mb: microbody, × 23,000.

Fig. 2. Control experiment of immunostaining. The *n*-alkane-grown *Candida tropicalis* cell was treated with non-immune rabbit IgG. It showed a few gold particles of non-specific reactions. CW: cell wall, × 23,000.

Fig. 3 Western blot analysis of T-I (A) and T-III (B) in the cell-free extract and P₂-S fraction. Anti-T-I antiserum prepared from purified T-I and anti-T-III antiserum prepared from purified T-III were used. The cell-free extracts obtained with the method reported previously (7) were electrophoresed (lane 1 in panel A, 130 μg; lane 1 in panel B, 8.7 μg). P₂-S fractions prepared from P₂ fraction as described previously (3) were also applied (lane 2 in panel A, 56 μg; lane 2 in panel B, 6.6 μg). Arrows indicate the bands of T-I (41 kDa) and T-III (43 kDa) subunits, respectively. Phosphorylase b (94.0 kDa), albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), and trypsin inhibitor (20.1 kDa) were used as markers.

In the cells embedded in Lowicryl K4M, many microbodies and the other organelles are also visible. Membranes appear as negative images through the use of aldehyde fixation, but mitochondrial cristae are also evident (Figs. 2,4-6). Negative control sections reactions reacted with non-immune rabbit IgG did not show any immunoreactivity (Fig. 2).

Figure 3 shows that there is no cross-reaction between anti-T-I and anti-T-III antisera. The reaction product with anti-T-I antiserum appeared at the position of 41 kDa, when the cell-free extract or P₂-S fraction was used. Bands below the arrowhead were considered to be the degradation products of T-I. The reaction product with anti-T-III antiserum appeared at the position of 43 kDa. In this case, no degradation products were observed.

The deposition of gold particles obtained with anti-T-I antiserum was observed in the cytoplasm (←) as well as in the microbodies (Fig. 4), while gold particles for anti-T-III antise-

Fig. 4-6.

Table I SUBCELLULAR LOCALIZATION OF T-I AND T-III IN *n*-ALKANE-GROWN *Candida tropicalis* CELLS.

Fraction	T-I	T-III (nmol·min ⁻¹)
P ₂	82 (63%)	561 (89%)
S ₂	48 (37%)	69 (11%)

P₂ and S₂ fractions were prepared as described in MATERIALS AND METHODS.

rum were mostly observed in the microbodies (Fig. 5). The reactions of anti-T-I and anti-T-III antisera were further analyzed by double-labeling technique using two different sizes of colloidal gold. Large particles of T-I (20 nm, ◀) and small particles of T-III (10 nm, ↔) were concurrently detected in a microbody and T-I was also observed in the cytoplasm (Fig. 6).

Analysis by immunoelectron microscopy confirmed the results of biochemical analysis based on subcellular fractionation (Table I). Cytoplasmic T-I is considered to have some relationship with sterol biosynthesis (unpublished data). The presence of both types of thiolases, T-I and T-III, in yeast microbodies was demonstrated by immunoelectron microscopy, which strongly indicates that the yeast β -oxidation system operates efficiently in microbodies alone.

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Fig. 4. Immunogold labeling of an *n*-alkane-grown *Candida tropicalis* cell with antiserum against T-I. Gold particles (10 nm) were observed in microbodies and cytoplasm. $\times 23,000$.

Fig. 5. Immunogold labeling of part of an *n*-alkane-grown *Candida tropicalis* cell with antiserum against T-III. Gold particles (10 nm) were localized in microbodies. $\times 38,000$.

Fig. 6. Part of an *n*-alkane-grown *Candida tropicalis* cell double immunogold labeled by two different sizes of gold particles. Large particles (20 nm, ◀) show the location of T-I and small particles (10 nm, ↔) the location of T-III. $\times 38,000$.