ULTRASTRUCTURE OF MICROBODIES OF
METHANOL-ASSIMILATING YEASTS

JIRO TSUBOUCHI,¹ KENZO TONOMURA, AND KENJI TANAKA*

Fermentation Research Institute, Inage, Chiba 281, and
*The Institute of Applied Microbiology, University of Tokyo,
Bunkyo-ku, Tokyo 113, Japan

(Received November 26, 1975)

The fine structure of yeasts capable of utilizing methanol as the sole source of carbon, Candida sp., Saccharomyces sp., and Torulopsis sp., was studied by electron microscopy. Many large microbodies were observed in clustered form in methanol-grown cells. They were observed as refractile granules under a phase contrast microscope. The membrane of the microbody was not stained by a method which specifically stains the plasmalemma, and an asymmetric distribution of the particles between the freeze-fractured half membranes was demonstrated. Microbodies in cells at the stationary phase of growth contained crystalline inclusions; crystalloids from Candida showed a tetragonal pattern, while those from Saccharomyces a hexagonal one. The development of microbodies was studied both during normal growth in the methanol medium and during the period of adaptation which occurs upon transfer from glucose to methanol medium. The possible modes of synthesis of the structure were discussed.

A yeast capable of assimilating methanol as the sole source of carbon and energy was first described by OGATA et al. (1). Since then many strains of yeast capable of growing on methanol have been isolated and the metabolism of methanol, its oxidation (2) and assimilation (3), have been studied. It has recently been established that methanol-utilizing yeasts when grown on methanol but not on glucose or ethanol have a special intracellular inclusion, a cluster of microbodies which contain alcohol oxidase as well as catalase (4–6). The microbodies in these yeasts were characterized by their relatively large size and an appearance in clustered form.

¹ Research trainee from Mitsubishi Gas Chemical Company, Inc.

In this paper we describe the ultrastructure of the microbodies in the methanol-assimilating yeast cells with respect to the membrane characteristics, crystalline inclusions, and their development.

MATERIALS AND METHODS

Organisms and cultures. The yeasts used were three species, Candida sp. N-16, Saccharomyces sp. H-1, and Torulopsis sp. M-1, which were isolated by Tonomura et al. (7) as the methanol-assimilating yeast. Yeast cells were grown in 500-ml Erlenmeyer flasks containing 150 ml of a mineral medium supplemented with vitamins and either methanol or glucose (1%, w/v), by shaking on a rotary shaker (220 rpm) for one to four days at 27°. The growth medium contained the following chemicals in grams per liter of deionized water: (NH₄)₂HPO₄, 3; KH₂PO₄, 1; MgSO₄·7H₂O, 0.4; FeSO₄·7H₂O, 0.01; CaCl₂·2H₂O, 0.01; MnSO₄·4H₂O, 0.002; Na₂MoO₄·2H₂O, 0.001; ZnSO₄·7H₂O, 0.001; yeast extract (Difco), 0.1; thiamine-HCl, 0.0004; biotin, 0.0002. Methanol was added after autoclaving.

Electron microscopy. Yeast cells were spun down and fixed immediately with 2.5% glutaraldehyde and 2% paraformaldehyde mixture in 0.05 M potassium phosphate buffer (pH 7.3) for 30 min. After the cells were washed in the same buffer for at least 3 hr or as long as 24 hr, they were fixed in 2% OsO₄ for 3 hr. The cells were washed twice in the same buffer, three times in deionized water, and soaked in 0.5% uranyl acetate in deionized water for 2 hr, followed by washing twice in water. All manipulations were carried out at room temperature. The cells, embedded in small agar blocks, were dehydrated through an alcohol series and were finally transferred to absolute acetone, and infiltrated with Spurr's low-viscosity epoxy resin (8). Polymerization was done by heating at 70° for 24 hr. Thin sections were obtained with glass knives on a Porter-Blum MT-2 ultramicrotome and stained in uranyl acetate solution and Reynolds' lead citrate (9).

In order to determine the nature of the microbody membrane, selective staining of plasma membrane was applied according to the method of Roland et al. (10). Thin sections were treated with periodic acid solution, and stained with phosphotungstic acid-chromic acid mixture.

To prepare the cells for freeze-fracturing, cells were fixed with a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer for 1 hr at room temperature and preserved in 30% or 40% (v/v) glycerol overnight. Freeze-fractured replicas were obtained with a block type of JEOL freeze-etching device installed in JEOL vacuum evaporator type 4C (11).

Sections and freeze-fractured replicas were examined with JEM 7A electron microscope operated at 80 kV.
RESULTS AND DISCUSSION

Phase contrast microscopy

Candida sp. N-16 cells in the logarithmic phase of growth taken from one-day culture were observed under a phase contrast microscope. Both cells grown on glucose and on methanol showed an oval shape of 2–3 μm by 3–8 μm in diameter, a morphology typical of yeast. Several refractile granules were found in the methanol-grown cells but not in the cells grown on glucose. The granules also appeared in young bud and clustered adjacent to the well-developed vacuole (Fig. 1). Saccharomyces sp. H-1 and Torulopsis sp. M-1 had refractile granules of the same kind in the cells cultured in the methanol medium.

Electron microscopy on the microbody

We were able to confirm the previous reports from other laboratories on the appearance of microbodies in the methanol-assimilating yeasts. Further studies revealed some ultrastructural aspects of this peculiar structure. Three species of yeasts capable of utilizing methanol as the sole source of carbon, Candida sp. N-16, Saccharomyces sp. H-1, and Torulopsis sp. M-1, generally showed a similar ultrastructure of cells in the logarithmic phase of growth. For convenience we studied mostly the structure of Candida sp. N-16. A section of a Candida sp. N-16 cell grown on glucose is shown in Fig. 2 and a section of a cell grown on methanol is shown in Fig. 3. Cells of Candida grown on methanol for one day had several microbodies with finely granular matrix and a dense core surrounded by a single limiting membrane (Fig. 3). The size and location of these bodies in the electron micrograph adjacent to the vacuole suggested that they correspond to the refractile bodies observed by the phase contrast microscopy. Microbodies were on the average about 0.5 μm in diameter, but those greater or smaller than 0.5 μm were often encountered. Up to eight bodies were counted per section. They were clustered and packed with their membranes pressed against each other, thus resulting in an angular shape (Fig. 3). Membranes of microbodies adjoining the vacuole were always separated from the vacuolar membrane at a distance of at least 10 nm (Fig. 4). Free surfaces on the cytoplasmic side had a wavy appearance.

The membrane of the microbody was a trilamellar structure about 7 nm thick, consisting of 2-nm parallel opaque lines separated by an interspace of 3 nm. These dimensions were the same for the vacuolar membrane. Freeze-replicas of methanol-grown Candida cells showed fractured face of the microbody membranes (Fig. 5). Concave face (A face) of the fractured membrane was covered with more particles than the convex face (B face) was. This observation does not agree with that of Dijken et al. (4), the only study on the freeze-replicas of methanol-grown yeasts reported to date. They described both the inner and outer fractured faces as smooth, while our results showed an asymmetric distribution of particles between the two faces. As far as the particles on the fractured half of the mem-
branes facing the cytoplasm (B face) are concerned, it can be said in qualitative terms that their population was similar in number to those found on the membrane of the plasmalemma (Fig. 7), vacuole, microbody (Fig. 6), and endoplasmic reticulum (Fig. 10).

The nature of the microbody membrane was studied by another method that stained the membranes originating from plasmalemma selectively. One of the results is shown in Fig. 8 in which only plasmalemma was stained intensely while other structures were not. This showed that membrane of the microbody is more akin to the cytoplasmic membrane systems and different from the plasmalemma.

It was frequently observed that a part of microbody was bordered by an endoplasmic reticulum, sometimes two membranes of which were pressed so tightly that it gave a five-layered structure (Fig. 9) and a dumbbell-shaped structure resulted (Figs. 9 and 10). Similar morphology was also found in cells of the logarithmic phase of Saccharomyces sp. H-1 and Torulopsis sp. M-1. This relationship between the two structures could represent an active involvement of endoplasmic reticulum in the development of the microbody.

**Crystalloids in the microbody**

Cells grown for 4 days into the stationary phase of growth had microbodies that included electron-dense crystalloids with a highly organized pattern (Fig. 11). With the development of the crystalline structures, the limiting membrane of microbodies seemed to be partly or to a large extent broken, and one of these structures together with membrane fragments was sometimes seen to be released into the lumen of the vacuole (Fig. 12). Number of microbodies in the stationary phase cell was observed to be decreased to one-half of that in the log phase cell. These crystalloids would be organized from the dense cores once observed in the matrix of the growing cells, which sometimes consisted of a few dense lines of regular pattern. Figures 13 and 14 are higher magnifications of crystalloid-containing microbodies of Candida sp. N-16 and Saccharomyces sp. H-1, respectively. Crystalloids in Candida cells showed a tetragonal pattern, in which repeated lines of 7 nm width with spacings of 14 nm crossed at right angles. Various aspects of sectioned crystalloids in Saccharomyces are shown in Fig. 14. A hexagonal pattern, which seemed to be a cross section of the structure, has dense lines of 7–8.5 nm width with spacings of the same width crossing each other at an angle of 60°. Another pattern, one of the longitudinal sections, consisted of the lines of 2 nm in repeating pattern with an interval of 7 nm. Crystalloids found in Torulopsis sp. M-1 resembled those seen in Candida sp. N-16 in their structural pattern.

Dense cores, or nucleoids in the microbody are widely known in plant and animal cells, and a classification of the types of cores was proposed (12). However, the type of the core is not specific; various types of crystalloid structure were found to occur in microbodies irrespective of tissues, organs, and species. Even within the same particles, found in the liver and kidney of the horse (Equus caballus),
different kinds of crystalloid pattern were observed (12). Occurrence of crystalloids in the microbodies of methanol-grown yeasts seems to depend on culture conditions (4) and the growth phase. Also the fixation method seems to be decisive; permanganate fixation failed to disclose the crystalline structure (5). From these facts, the failure to demonstrate crystalloids in the profusely occurring microbodies in the hydrocarbon-utilizing yeast needs further investigation. Our findings that different patterns of crystalloids have been observed in the microbodies of supposedly the same function from different species of the methanol-utilizing yeasts could be of importance in considering the significance which crystalloid pattern bears.

**Development of microbodies**

Development of microbodies in the methanol-grown yeasts was studied in the two processes; one is in the budding of the cell in the logarithmic phase of growth and the other in the period of adaptation by a transfer of the glucose-grown cells to the medium containing methanol.

First, there is the problem of how and where microbodies in the growing bud come into appearance during proliferative growth of yeast cells in the methanol medium. A cell at an early stage of budding showed inconspicuous bodies less than 0.2 μm in diameter with membranes indistinctly bounded; one in the bud and others in close apposition to mature microbodies (Fig. 15). At a more advanced stage in the budding, a daughter cell had a number of microbodies with a dense core, but of smaller size than those seen in the mother cell (Fig. 16). These small bodies, tentatively assumed to be premature microbodies, could be derived from extensions and protrusions of the membrane of a microbody synthesized. **Osumi et al.** (13) proposed the division of a preexisting microbody during its multiplication in the hydrocarbon-grown yeasts. However, the process in this study leading to the formation of small microbodies from larger ones does not necessarily mean a process of division in an ordinary sense, but seems rather to be a process of fragmentation or budding from the preexisting structures, as suggested by **Legg and Wood** (14).

Secondly, development of microbodies was followed in a more direct way during the period of adaptation in the transfer from the culture in the glucose medium to that in the methanol one. When **Candida sp. N-16** cells cultured for one day on glucose medium were washed several times with deionized water and transferred to the methanol medium, growth in the new medium was observed to begin, as measured by an increase of optical density, after a lag period of adaptation of 16 hr, during which aliquots were taken for electron microscopy. Cells grown on methanol for 2 hr had few structures equivalent to the microbodies, and 5-hr cells, several particles recognizable as microbodies limited by a membrane but of smaller size and not so tightly packed against each other. Some of them contained a dense core within the particle. Frequently one encountered in the cyto-
plasm several islands with no limiting membrane which excluded ribosomes with amorphous bodies, very similar in appearance to the dense cores seen in the adjoining microbodies (Fig. 17), and on rare occasions they were seen to be partly enclosed by a membrane, which might have originated from the endoplasmic reticulum (Fig. 18). In 8-hr cultured cells microbodies carrying dense cores appeared in a cluster, though still not in a packed state (Fig. 19).

These observations suggest the process of microbody formation in the methanol-utilizing yeasts as follows: Proteins or enzymes specific for the microbody synthesized on ribosomes in the cytoplasm would be assembled to form amorphous bodies, which are destined to be enclosed by a developing membrane from a kind of endoplasmic reticulum. Once established as particulate structures, microbodies could increase in size with concomittant modification of the limiting membrane as well as the included proteinaceous material. A process that mature microbodies would develop from "precursor particle of low buoyant density" was indicated by Feierabend and Beevers in their biochemical studies on peroxisomes of greening leaves (15).

Many workers have discussed the origin of the microbodies and favor the view that the structure comes from the endoplasmic reticulum, since it is frequently observed that the microbody comes out of the dilated area of the endoplasmic reticulum in animal and plant cells (12). No one has made such kind of observations on microbodies in yeast cells grown either on methanol (5) or on hydrocarbon (13). Of course it is a characteristic of the yeast cell that the endoplasmic reticulum is poorly developed. We have shown here in the studies during the period of adaptation from glucose-grown to methanol-grown cells some figures that suggest a kind of de novo formation of the structure, whose limiting membrane could be developed from endoplasmic reticulum in the cytoplasm. Growth or increase in size of the microbody seems to proceed gradually without concomittant increase of their number in the case of methanol-grown cells.

REFERENCES


**EXPLANATIONS OF THE FIGURES**

Key to Abbreviations. ER, endoplasmic reticulum; M, mitochondrion; MB, microbody; N, nucleus; PM, plasmalemma; V, vacuole.

Bar represents 0.5 μm in electron micrographs, unless otherwise indicated. All figures except Fig. 14 are of Candida sp. N-16.

Fig. 1. Phase contrast micrograph of yeast cells grown for 1 day in the methanol medium. Microbodies are indicated by arrows.

Fig. 2. Section of a cell grown on glucose medium for 1 day.

Fig. 3. Section of a methanol-grown cell. Eight microbodies are in a cluster.

Fig. 4. Portion of a section of a methanol-grown cell, showing ultrastructure of microbody and vacuole membranes. Note the distance between the membranes of the microbody and vacuole. A part of membranes of adjoining microbodies showed five-lamellar structure (an arrow).

Figs. 5, 6, 7. Freeze-fractured replicas, showing the fractured faces of membranes. ~ and ~ indicate the convex and concave face, respectively.

Fig. 8. Section of a cell grown on methanol, treated by the selective staining method, only plasmalemma being stained.

Fig. 9. Portion of a section of a methanol-grown cell. Endoplasmic reticulum borders a microbody. There is a membrane vesicle between the microbodies (an arrow).

Fig. 10. A freeze-fractured replica, showing a similar morphology to Fig. 9.

Fig. 11. Section of a cell cultured for 4 days in the methanol medium. Density of the cores increased and one of them seems to be degraded.

Fig. 12. Portion of a section of a methanol-grown cell cultured for 4 days. A degrading microbody, releasing a crystalloid and the membrane (an arrow) into the lumen of the vacuole.

Fig. 13. Crystalloids developed in microbodies from 4-day cultured Candida sp. N-16, showing the tetragonal pattern.

Fig. 14. Crystalloids in microbodies from 4-day culture of Saccharomyces sp. H-1, which have a hexagonal structure.

Fig. 15. Portion of a section of an actively growing cell, showing the moving microbodies with indistinct boundaries in the growing bud.

Fig. 16. Microbodies seen in a daughter cell.

Fig. 17. Portion of a section of a cell cultured for 5 hr after transfer from glucose to methanol medium. Dense amorphous bodies without boundaries (arrows) are seen.

Fig. 18. Portion of a section of a cell from the same culture as in Fig. 17. A membrane is enclosing the dense material.

Fig. 19. Portion of a cell cultured for 8 hr in the methanol medium after transfer from the glucose medium. A cluster of microbodies is seen.
Microbodies of Methanol-assimilating Yeasts