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Development of Multipurpose Peroxisomes in *Candida boidinii* Grown in Oleic Acid-Methanol Limited Continuous Cultures

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We have studied the development and metabolic significance of peroxisomes in the yeast *Candida boidinii* following adaptation of the organism to cultivation conditions which require the simultaneous presence and activity of two independent peroxisome-mediated pathways for growth. After the addition of methanol to oleic acid-grown cells at late exponentional growth, a number of new small peroxisomes developed which, apart from the presence of β-oxidation enzymes, were characterized by the presence of enzymes involved in methanol metabolism (alcohol oxidase and dihydroxyacetone synthase). The latter proteins, however, were absent in the larger organelles which were originally present in the oleic acid-grown cells prior to the addition of methanol and which contained only enzymes of the β-oxidation pathway. Subsequent experiments on cells from continuous cultures grown on a mixture of oleic acid and methanol at steady-state conditions revealed that both the enzymes of the β-oxidation pathway and those involved in methanol metabolism were found in one and the same compartment. Thus, under these conditions the cells contained peroxisomes which were concurrently involved in the metabolism of two different carbon sources simultaneously used for growth. Our results indicated that the heterogeneity in the peroxisomal population of a single cell, observed in the transient state following the addition of methanol, is only temporary and due to heterogeneity among these organelles with respect to their capacity to incorporate newly synthesized matrix proteins.

In yeasts, microbodies (peroxisomes and glyoxysomes) play an essential role in the metabolism of different carbon and/or organic nitrogen sources (21, 22). Initially, it was thought that the organelles were of minor metabolic significance and contained only one or more catalytic functions. The finding, however, that microbodies present in methylotrophic yeasts contain not only alcohol oxidase, the key enzyme of methanol metabolism, but also dihydroxyacetone synthase (4, 7), a key enzyme in carbon assimilation under these conditions (18), together with other biosynthetic enzymes (28) stresses the importance of these organelles and clearly places their metabolic significance beyond that of a compartment purely involved in catabolism.

Yeast microbodies do not arise de novo but develop by growth and fission of existing organelles (22, 23); however, experiments performed with the methylotrophic yeast *Hansenula polymorpha* indicated that the capacity of individual organelles to incorporate newly synthesized matrix proteins may be related to their developmental stage (24).

The recent finding that, in the methylotrophic yeast *Candida boidinii*, massive microbody proliferation may be induced by carbon sources other than methanol (9, 16) makes it possible to study the development and functioning of the microbodies during the growth of cells in media supplemented with more than one carbon source, each of which is metabolized via specific peroxisome-mediated metabolic pathways. The present study addresses two major questions. (i) In *C. boidinii*, is the capacity of individual microbodies to incorporate newly synthesized matrix proteins only temporary and related to the developmental stage of the organelles, as is the case in *H. polymorpha*? (ii) Are the different key enzymes that are involved in the metabolism of two different carbon sources simultaneously used for growth found in the same or in different microbodies?

We studied these questions by (i) following the fate of the microbodies present in cells after the addition of methanol to cells grown in batch cultures on oleic acid and (ii) studying their enzyme composition in cells grown in continuous cultures on a mixture of oleic acid and methanol. The results of these studies are presented in this paper.

MATERIALS AND METHODS

Organism and growth. *C. boidinii* (ATCC 32195) was grown at 30°C in batch cultures on 0.1% (vol/vol) oleic acid as described previously (16). In the late exponential growth phase, when oleic acid was not yet fully used up, methanol (0.5% [vol/vol] final concentration) was added to the cultures. Samples were taken at 0, 2, 4, 6, and 8 h after the addition of methanol.

In addition, cells were grown at 30°C in carbon-limited continuous cultures at a dilution rate (D) of 0.07 h^{-1} in the mineral medium described previously (18). The medium contained two carbon sources, namely, 0.1% (vol/vol) oleic acid (in the presence of 0.05% [vol/vol] Tween 80) and 0.25% (vol/vol) methanol. In order to obtain such cultures, cells were first grown on oleic acid alone. After the culture had reached a steady state (at D = 0.07 h^{-1}), 0.25% (vol/vol) methanol (final concentration) was added to the medium vessel. Samples were taken from steady-state cultures grown on 0.1% (vol/vol) oleic acid–0.25% (vol/vol) methanol; monitoring of both oleic acid and methanol concentrations indicated that their respective concentrations were undetectably low in the culture vessel of steady-state cul-

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tures, indicating that these mixed-substrate cultures grew under carbon limitation.

Biochemical methods. To prepare crude extracts, cells were washed twice in 50 mM potassium phosphate buffer (pH 7.0; 0°C) and subsequently resuspended in the same buffer. After the addition of one-third volume of acid-washed glass beads, the mixture was spun for 3 min at 4°C in a Vortex and the homogenate was centrifuged for 15 min at 4°C in an Eppendorf Microfuge (12,000 × *g*). The resulting supernatant is referred to as crude extract. Alcohol oxidase (AO) (27), catalase (12), 3-oxoacyl-coenzyme A (CoA) thiolase (10), multifunctional enzyme (determined as combined enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activity) (13), and cytochrome *c* oxidase (4) were determined by established methods. Protein concentrations were determined as described by Bradford (1), using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (11); gels were stained with Coomassie brilliant blue. Western blotting (immunoblotting), using specific polyclonal antibodies against alcohol oxidase, dihydroxyacetone synthase, and thiolase and monoclonal antibodies against two peroxisomal membrane proteins (PMP20 and PMP47) (8), was performed with the Protoblot immunoblotting system (Promega Biotec).

Cell fractionation methods. Protoplasts were prepared from cells grown in continuous cultures ($D = 0.07\ h^{-1}$) on 0.1% oleic acid–0.25% methanol and homogenized in a Potter homogenizer, and the obtained lysates were subjected to differential centrifugation as described previously (4). The advantage of this procedure is that the bulk of the mitochondria is removed in the initial centrifugation steps at 5,000 × *g* and 12,500 × *g* (16). The subsequent 30,000 × *g* pellet is highly enriched in peroxisomes, which were further purified by sucrose gradient centrifugation (4, 16).

Electron microscopy. Whole cells were fixed in 1.5% (wt/vol) $KMnO_4$ for 20 min at room temperature. Spheroplasts were fixed in 6% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 60 min at 0°C and were postfixed in a mixture of 0.5% (wt/vol) OsO_4 and 2.5% (wt/vol) $K_2Cr_2O_7$ in the cacodylate buffer for 90 min at 0°C. After dehydration in a graded ethanol series, the samples were embedded in Epon 812; ultrathin sections were cut with a diamond knife and examined in a Philips EM 300 or CM10.

(Immuno)cytochemistry. Cytochemical staining experiments for the detection of catalase activity were performed by the 3,3-diaminobenzidine (DAB)-based method (19). For the detection of AO activity, a previously described $CeCl_3$ method (26) was slightly modified to account for the fact that AO activity in spheroplasts of *C. boidinii* appeared to be extremely sensitive to aldehyde prefixation. Glutaraldehyde could not be used, since this compound completely abolished AO activity within a 5-min time interval. Activities of the enzyme could only be demonstrated after a very mild fixation in 0.5% formaldehyde solution for 15 min at 0°C; under these conditions, approximately 75% of the original AO activity was recovered. Since spheroplasts were not osmotically stable after this treatment, the incubations with $CeCl_3$ and methanol were performed in the presence of 0.7 M sorbitol as an osmotic stabilizer. Postfixation and subsequent embedding were performed as described previously (26).

For immunocytochemistry, intact cells were fixed in 3% (vol/vol) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 90 min at 0°C, dehydrated in a graded ethanol series, and embedded in Lowicryl K4M (4). Immunolabeling was performed on ultrathin sections with specific antibodies against AO, dihydroxyacetone synthase, thiolase, PMP20, and PMP47 (4, 5, 7, 8) by the protein A-gold method described by Slot and Geuze (15).

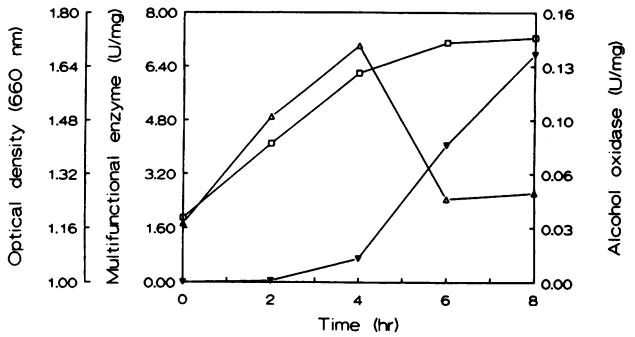

FIG. 1. Growth (expressed as optical density at 660 nm) and activities of multifunctional enzyme (combined enoyl-CoA-hydratase and L-3-hydroxyacyl-CoA dehydrogenase activity) and AO at times (hours) after the addition during late exponential growth phase of 0.5% (vol/vol) methanol to a batch culture of *C. boidinii* grown on oleic acid. Symbols: □, optical density at 660 nm; △, multifunctional enzyme; ▼, AO.

RESULTS

Growth and enzyme kinetics. Growth and enzyme activities of multifunctional enzyme and AO (key enzymes of oleic acid and methanol metabolism, respectively) after the addition during late exponential growth phase of methanol to a batch culture of *C. boidinii* grown on oleic acid are shown in Fig. 1. After the addition, growth continued, although at a reduced rate (the doubling time increased from 3 h on oleic acid alone to approximately 7 h on the mixture). Adaptation of the cells to the new environment was associated with an initial increase in multifunctional enzyme activity, which subsequently dropped to original levels (Fig. 1). AO activity was not detectable in the oleic acid-grown cells, but its activity was gradually induced after the addition of methanol.

The induction of AO protein was confirmed by Western blotting experiments (Fig. 2). Two other microbody-borne enzymes associated with methanol metabolism, namely,

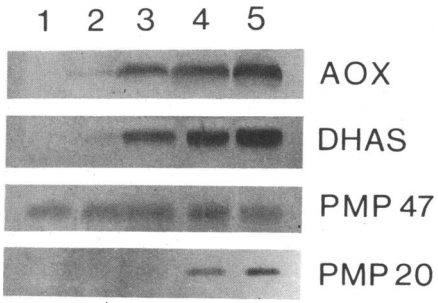

FIG. 2. Western blots of crude extracts of cells of *C. boidinii* grown on oleic acid from late exponential growth phase (lane 1) and after exposure to excess methanol for 2 (lane 2), 4 (lane 3), 6 (lane 4), and 8 h (lane 5). Each lane was loaded with 5 μg of protein. AOX, alcohol oxidase; DHAS, dihydroxyacetone synthase; PMP 47, peroxisomal membrane protein of 47 kDa; PMP 20, peroxisomal membrane protein of 20 kDa.

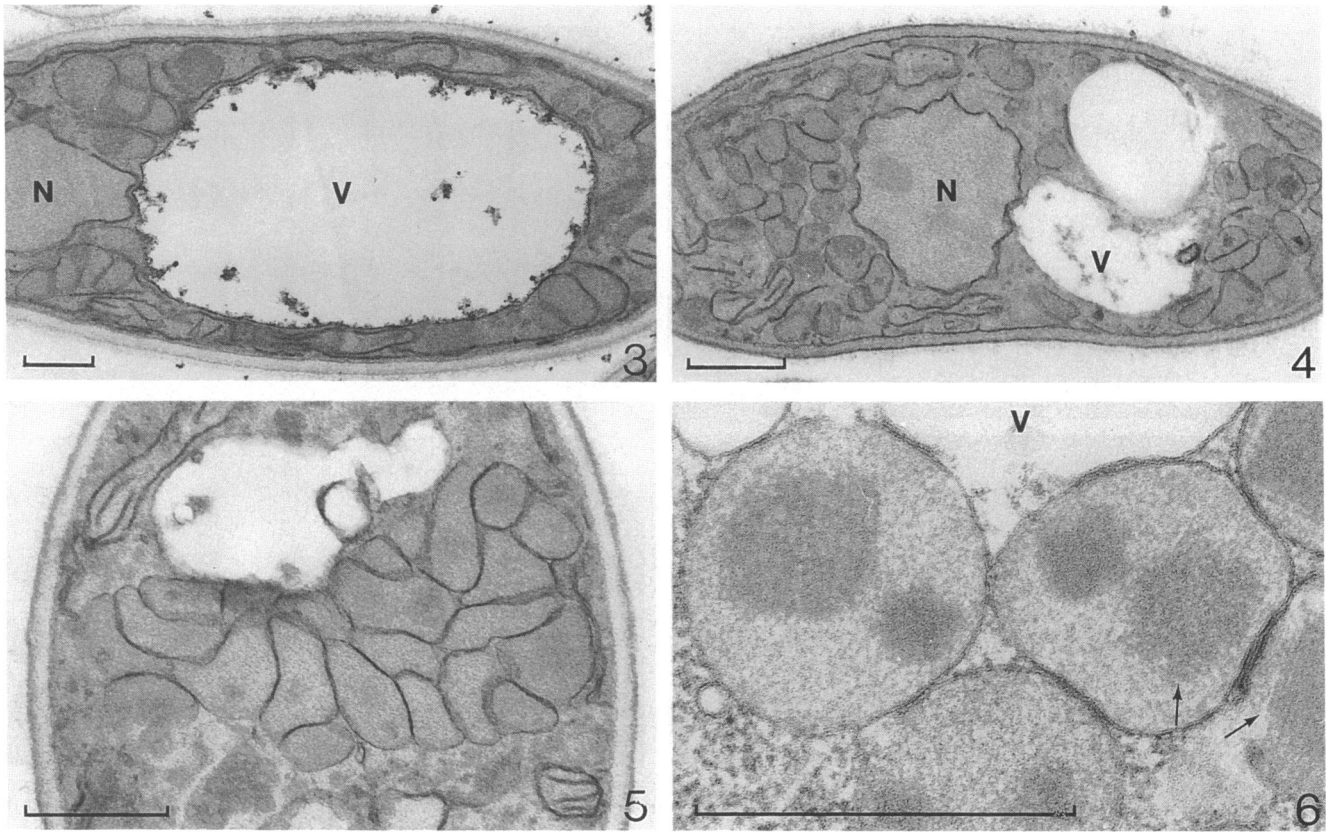

FIG. 3–6. Overall cell morphology and induction of peroxisomes in cells from batch cultures grown on oleic acid (Fig. 3) and 2 h (Fig. 4) or 6 h (Fig. 5) after the addition of methanol. Eight hours after the addition of methanol (Fig. 6), the matrix of part of the organelles contains crystalloids of AO (arrows), together with the dense cores characteristic of organelles from oleic acid-grown cells (fixation, glutaraldehyde-OsO_4). Owing to the oblique section, the crystalline structure of the crystalloid in the peroxisome at the left of Fig. 6 is not resolved. Unless otherwise stated, all electron micrographs are of intact cells of *C. boidinii* fixed in $KMnO_4$. Abbreviations: M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bars, 0.5 μm.

dihydroxyacetone synthase and a protein of as yet unknown function termed PMP20 (8), showed similar induction patterns. The levels of PMP47, a constitutive integral peroxisomal membrane protein (9), did not vary significantly after the addition of methanol (Fig. 2).

Ultrastructural analysis. In *C. boidinii* cells growing exponentially on oleic acid, several clusters of elongated and often interwoven microbodies (peroxisomes) are present (Fig. 3). These may contain electron-dense cores and are characterized by the presence of catalase and enzymes of the β-oxidation pathway (16). After the addition of methanol to such cultures in late exponential growth phase, the number of peroxisomes gradually increased (Fig. 4). The newly formed peroxisomes showed a more rounded form and developed by fission from already existing organelles by previously described mechanisms for the shift of cells from glucose to methanol (20). Six hours after the addition, the cells characteristically contained one or two large clusters, consisting of numerous peroxisomes (Fig. 5). Part of these organelles contained electron-dense cores together with small crystalloids, as was revealed by glutaraldehyde-OsO_4 fixation (Fig. 6). On the basis of cytochemical staining experiments, AO activity was localized in only a few of the organelles, which were present in the cluster during the initial hours after the addition (Fig. 7). Both the size and number of the organelles increased with increasing incubation times; after 8 h of incubation, the AO-containing organelles represented approximately half of the total peroxisomal population present in individual cells. Immunocytochemically, AO protein showed identical distribution patterns (Fig. 8), indicating that the absence of enzyme activity in part of the organelle population indeed has to be ascribed to the absence of the enzyme. The two other inducible proteins studied, dihydroxyacetone synthase (Fig. 9) and PMP20 (not shown), showed distribution patterns identical to that of AO (Fig. 8). After experiments performed with specific antibodies against thiolase, all peroxisomal profiles present in the cell were labeled (Fig. 11); in addition, all organelles harbored catalase activity, as was evident after cells were incubated with DAB-H_2O_2 (Fig. 12). Interestingly, concomitant with the induction of peroxisomes, large cytosolic protein aggregates may develop during the initial hours after the addition of methanol. These structures displayed neither catalase nor AO activity as judged from cytochemical experiments, but they were strongly labeled after immunocytochemical experiments using antibodies against AO protein and protein A-gold (Fig. 10). Identical labeling patterns (although at reduced intensities) were observed when specific antibodies against dihydroxyacetone synthase or thiolase were used in these experiments (not shown). Preliminary ultrastructural observations suggested that these protein aggregates were gradually degraded at later stages of incubation by uptake in the vacuole. Since microbodies are known to develop from preexisting organelles, the develop-

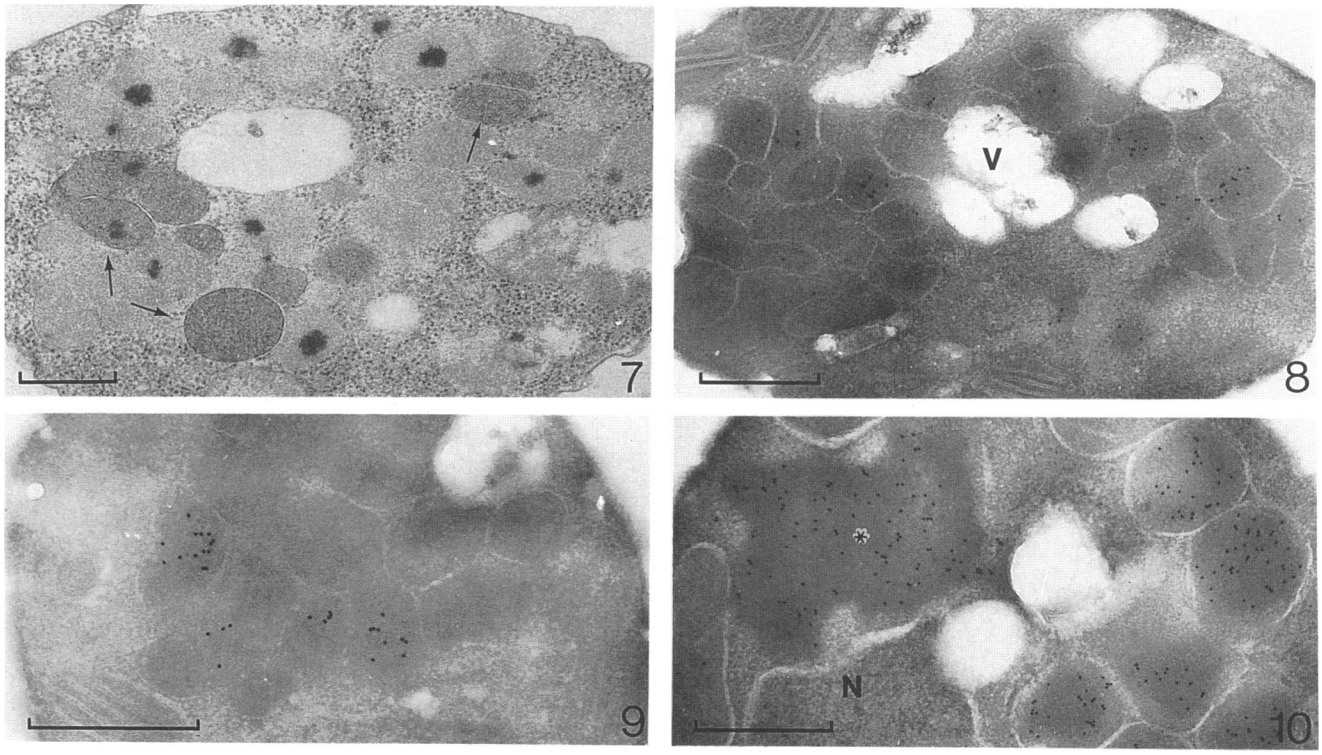

FIG. 7. Cytochemical demonstration of AO activity (arrows) in part of the peroxisomal population after incubation of cells for 4 h in the presence of methanol. Fixation, glutaraldehyde-OsO_4.

FIG. 8–10. Immunocytochemical demonstration of AO (Fig. 8) and dihydroxyacetone synthase (Fig. 9) by using specific antibodies against these proteins and protein A-gold on sections of cells incubated for 4 h in the presence of methanol. Figure 10 shows labeling (by using antibodies against AO) of peroxisomal profiles and a large cytosolic proteinaceous aggregate in cells incubated for 8 h after the addition of methanol. Fixation, glutaraldehyde-uranyl acetate. Abbreviations and bars are as in Fig. 3–6.

ment of organelles in transient-state cells that contain both thiolase and AO (as judged from immunocytochemistry; see Fig. 9 and 11) is the expected result (16, 20). However, their presence at this stage of growth does not exclude the possibility that in steady-state cells of *C. boidinii* grown on oleic acid-methanol mixtures, two types of peroxisomes may exist which are involved in the metabolism of either oleic acid or methanol. To address this question, cells from carbon-limited continuous cultures using a growth medium containing these two carbon sources were analyzed. Cells of steady-state cultures ($D = 0.07\ h^{-1}$) contained numerous microbodies, which were significantly smaller than those from cells grown on methanol alone (20) and were organized into one or two large clusters (Fig. 13). The organelles were predominantly rectangular and often highly elongated (inset, Fig. 13). All of these organelles displayed catalase and AO activities, as was revealed by cytochemical methods (not shown). Thiolase and AO protein were localized in each organelle, as was evident from double-immunolabeling experiments (Fig. 14).

Cell fractionation. In order to further verify that, in steady-state cells, the enzymes of the β-oxidation pathway and AO were located in one organelle population, fractionation studies were performed. The final 30,000 × *g* pellet resulting from differential centrifugation of lysates of protoplasts of *C. boidinii* from continuous cultures was subjected to sucrose gradient centrifugation. In the gradient, one major protein peak was observed at approximately 53% sucrose. Biochemical analysis of the fractions obtained (Fig. 16) revealed that the bulk of the catalase activity, together with AO and enzymes of the β-oxidation pathway (multifunctional enzyme and thiolase), had sedimented in this protein peak at 53% sucrose. The mitochondrial contamination in the gradient was very low, as judged from cytochrome *c* oxidase activity (Fig. 16). As was observed in earlier experiments on oleic acid-grown *C. boidinii* (16), the bulk of these organelles had sedimented in the initial steps of the differential centrifugation procedure employed prior to sucrose gradient centrifugation. Electron microscopy confirmed that the protein peak at 53% sucrose (fraction 4) contained highly purified peroxisomes (Fig. 15).

DISCUSSION

In this paper, we provide evidence that in *C. boidinii* microbodies (peroxisomes) may develop which are concurrently involved in the metabolism of two different carbon sources (oleic acid and methanol). Our present findings are in accordance with the current views on microbody development and functioning in yeasts. All evidence obtained so far indicates that, in these organisms, microbodies develop from preexisting organelles by fission (20, 24). They contain enzymes involved in the metabolism of the carbon and/or organic nitrogen source(s) used for growth (16, 20–22, 25, 28). This implies that at steady-state conditions, all individual microbodies present in one yeast cell contain the same set of matrix enzymes. However, the organelles described in this study represent the first example of peroxisomes which

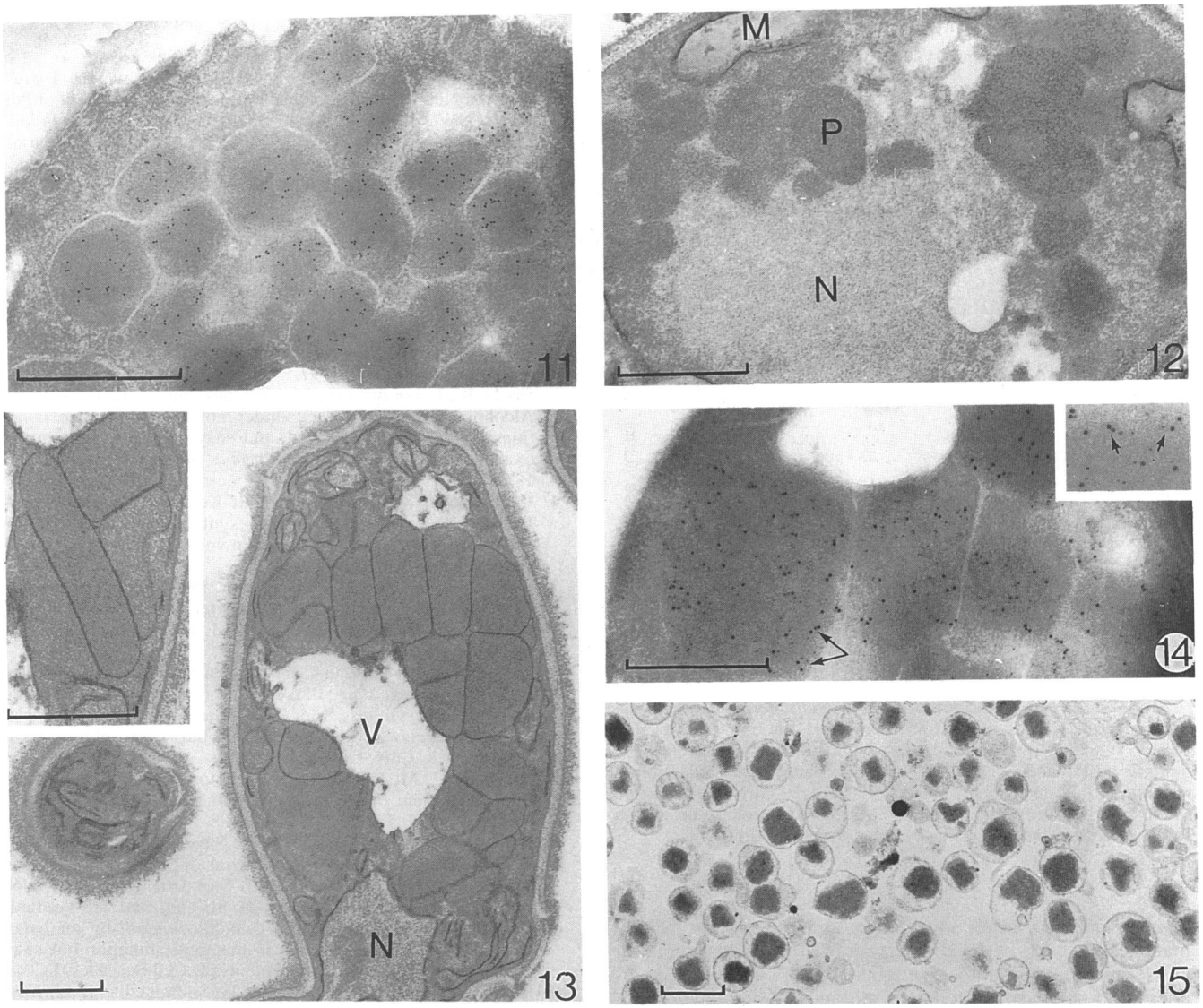

FIG. 11–15. Figure 11 is an immunocytochemical demonstration of the presence of thiolase protein in all peroxisomal profiles visualized in a thin section of a cell, 8 h after the addition of methanol to oleic acid-grown cells, by using specific antibodies against thiolase and protein A-gold (fixation, glutaraldehyde-uranyl acetate). The organelles also contained catalase activity (Fig. 12; DAB-H_2O_2). Figure 13 shows the overall morphology of cells grown in continuous culture on oleic acid-methanol. Peroxisomes are present in large clusters and often show a rectangular and/or elongated shape (inset, Fig. 13). After incubations of ultrathin sections of these cells with antibodies against AO (indicated by small 5-nm gold particles) and thiolase (indicated by large 12-nm gold particles), all individual organelles were double labeled (Fig. 14; fixation, glutaraldehyde-uranyl acetate). The inset shows a higher magnification of the parts (indicated by arrows). Figure 15 shows purified peroxisomes in the microbody peak fraction at 53% (wt/vol) sucrose (fraction 4, Fig. 16; fixation, glutaraldehyde-OsO_4). Abbreviations and bars are as in Fig. 3–6.

harbor the key enzymes of the metabolism of two different carbon sources simultaneously used for growth.

Microbodies with different sets of matrix enzymes have also been described for another methylotrophic yeast, *H. polymorpha*. They occur during the transient state, when cells adapt to a new growth environment (24). The observed response of *C. boidinii* to the addition of methanol to medium containing oleic acid is fully consistent with results obtained after a shift of cells from glucose to methanol (20) in that methanol induces a strong peroxisome proliferation. The fact that newly synthesized AO is not incorporated in all microbodies that are present in the oleic acid-grown cells after the addition of methanol is most probably not related to a limiting capacity of protein storage for these organelles. This view is based on findings, obtained with overproducing transformants of *H. polymorpha* (14) and *Saccharomyces cerevisiae* (6), which indicated that peroxisomes have a considerably higher storage capacity than is normally utilized in wild-type cells (14). Therefore, our results suggest that, in yeasts in general, heterogeneity may exist among organelles present in single cells with respect to their capacity to incorporate newly synthesized matrix proteins. In yeast cells growing at steady-state conditions, only a minor part of the microbody population is involved in organelle biosynthesis (development and multiplication); this property is unique for these organelles and has not been

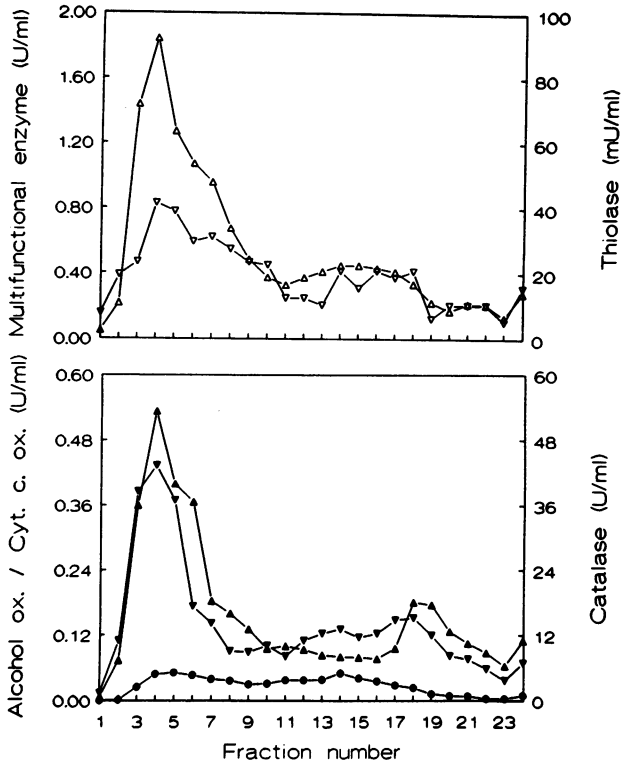

FIG. 16. Enzyme distribution after discontinuous sucrose gradient centrifugation of a microbody-enriched fraction (30,000 × *g* pellet), obtained by differential centrifugation of homogenized *C. boidinii* protoplasts. Protoplasts were prepared from cells from a continuous culture at steady state ($D = 0.07\ h^{-1}$) grown on a mixture of oleic acid and methanol. Symbols: △, multifunctional enzyme; ▽, thiolase; ▲, catalase; ▼, AO; ●, cytochrome *c* oxidase.

demonstrated for other subcellular organelles. How the cell manages to select between different organelles as targets for newly synthesized matrix proteins is still unknown. Experiments on peroxisome-deficient mutants of *H. polymorpha*, which have recently been isolated (2, 17), revealed that fission is not related to the size of the organelles.

A characteristic feature, observed only in the initial hours after the shift to methanol of *C. boidinii* cells pregrown on either oleic acid (this article) or glucose (20a), includes the formation of large cytosolic aggregates, consisting of microbody matrix proteins. There are different reasons to assume that this temporary failure of peroxisomal protein import is related to a misbalance in the rate of protein synthesis versus uptake. First, as indicated above, yeast peroxisomes in wild-type cells do not fully utilize their protein storage capacity at normal physiological conditions. Furthermore, expression of AO in *H. polymorpha* under nonmethylotrophic conditions (during growth on glucose) showed that the possible factors controlling import, assembly, and activation of the enzyme are constitutively present (3). Therefore, the finding that, besides AO, dihydroxyacetone synthase and thiolase protein are also present in the aggregates in *C. boidinii* suggests that in the early adaptation period, competition may exist for a common recognition and/or translocation machinery. In this respect, it is relevant to mention that methanol-induced microbody proliferation in *C. boidinii* is preceded by adaptations of the microbody membrane (20).

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