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An electron microscopical study of the development of peroxisomes during formation and germination of ascospores in the methylotrophic yeast *Hansenula polymorpha*

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VEENHUIS, M., KEIZER-GUNNINK, I. and HARDER, W. 1980. An electron microscopical study of the development of peroxisomes during formation and germination of ascospores in the methylotrophic yeast *Hansenula polymorpha*. *Antonie van Leeuwenhoek* 46: 129–141.

Ascospore formation was studied in liquid cultures of the yeast *Hansenula polymorpha*, previously grown under conditions in which the synthesis of alcohol oxidase was repressed (glucose as growth substrate) or derepressed (methanol, glycerol and dihydroxyacetone as growth substrates and after growth on malt agar plates). In ascospores obtained from repressed cells, generally one small peroxisome was present. The organelle probably originated from the small peroxisome, originally present in the vegetative cells. They had no crystalline inclusions and cytochemical experiments indicated the presence of catalase, urate oxidase and amino acid oxidase activities in these organelles. In ascospores obtained from derepressed cells, generally 1–3 crystalline peroxisomes were observed. These organelles also originated from the peroxisomes originally present in the vegetative cells by means of fragmentation or division. They contained, in addition to the enzymes characteristic for peroxisomes in spores from repressed cells, also alcohol oxidase. The latter enzyme is probably responsible for the crystalline substructure of these peroxisomes.

Peroxisomes had no apparent physiological function in the process of ascosporeogenesis. A glyoxyosomal function of the organelles during germination of the ascospores was also not observed. Germination of mature ascospores in media containing different sources of carbon and nitrogen showed that the function of the peroxisomes present in ascospores of *Hansenula polymorpha* is probably identical to that in vegetative haploid cells. They are involved in the oxidative metabolism of different carbon and nitrogen sources. Their enzyme profile is a reflection of that of peroxisomes of vegetative cells and their presence may enable the formation of cells which are optimally adapted to environmental conditions extant during spore germination.

INTRODUCTION

The occurrence of peroxisomes in cells of the ascomycetous yeast *Hansenula polymorpha* during different stages of vegetative reproduction is now well documented. It has been shown that the presence of these organelles and their physiological function is characteristically associated with the oxidative metabolism of a particular carbon source (van Dijken, Otto and Harder 1976; Veenhuis, Keizer and Harder, 1979) or nitrogen source (Zwart et al., in press). Furthermore, the number and substructure of the peroxisomes was dependent on the nature of the source of carbon or nitrogen used and varied with growth conditions of the cells (Fukui et al., 1975; Veenhuis et al., 1979; Eggeling and Sahm, 1978; Egli et al., 1980).

In contrast, the occurrence and – possible – function of peroxisomes during sexual reproduction in *Hansenula polymorpha* and of other ascomycetous yeasts is unknown. In filamentous fungi observations have been made which indicate a significant role of peroxisomes during the sporulation process (Cole, 1973; Hess and Weber, 1974). In addition, these organelles may have a possible function during germination of spores (Powell, 1976; Murray and Maxwell, 1974; Chong and Barr, 1973), similar to the function of peroxisomes in developing seeds (Hutton and Stumpf, 1969; Tolbert, 1971; Gerhardt, 1973).

As part of our studies on the development and physiological function of peroxisomes in yeasts we investigated the possible occurrence and function of peroxisomes during sexual reproduction in the yeast *Hansenula polymorpha*. This paper describes the development of peroxisomes in ascospores in relation to different conditions of growth and their behaviour during germination of spores in media supplemented with different sources for carbon and nitrogen.

MATERIALS AND METHODS

Organism and growth conditions

Hansenula polymorpha de Morais et Maya CBS 4732 was used in all experiments. Sporulating cultures were obtained by growing the organism a) in small Petri dishes on 1–5% malt extract agar (prepared from malt extract of 15° Balling) for 3–5 days at 37°C; b) in 500 ml shake flask cultures at 37°C in 200 ml medium containing per liter: NaH_2PO_4 , 3.0 g; K_2HPO_4 , 0.7 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; trace elements according to Vishniac and Santer (1957), 1 ml and distilled water. As nitrogen source 0.025% (w/v) Na-urate was added to this medium, while glucose, methanol, glycerol and dihydroxyacetone (all at 0.5% (w/v)) were used as carbon sources. The shake flask cultures were inoculated to an optical density (OD_{663}) of 0.005 with cells exponentially growing in media supplemented with one of the above mentioned carbon sources and $(\text{NH}_4)_2\text{SO}_4$ (1.5 g/l) as the nitrogen source; growth ceased at OD_{663} of 1.0–1.5. Spores were formed after 2–4 days of incubation.

Germinating spores were obtained by inoculating ascospores to an OD_{663} of 0.2 in 500 ml shake flasks containing 100 ml of the basal medium, described above. This medium was supplemented with 0.15% $(NH_4)_2SO_4$ and 0.5% glucose or 0.5% methanol or with 0.5% glucose and 0.2% methylamine as the nitrogen source instead of ammoniumsulphate. In addition, ascospores were inoculated in media lacking the source of carbon or nitrogen, respectively, or in tap water.

Preparation of spheroplasts

Spheroplasts were prepared by treatment of suspensions of whole cells with "Zymolyase" (Kitamura, Kaneda and Yamamoto, 1971) for 15 min at 37°C according to the procedure of Osumi et al. (1975).

Cytochemical staining techniques

Cytochemical staining procedures for the demonstration of catalase, alcohol oxidase, urate oxidase and amino acid oxidase activities were performed as described previously (Veenhuis et al., 1976). Malate synthase activity was determined with the copper-ferricyanide method according to Trelease, Becker and Burke, (1974).

Fixation and postfixation

Whole cells were fixed – or postfixed after the cytochemical staining procedures – with 1.5% $KMnO_4$ for 20 min at room temperature. Spheroplasts were fixed for 30 min in 6% glutaraldehyde in 0.1 M Na-cacodylate buffer pH 7.2 at 0°C and postfixed for 45 min in a solution of 2.5% $K_2Cr_2O_7$ and 1% OsO_4 in 0.1 M Na-cacodylate buffer pH 7.2. After dehydration in a graded alcohol series the cells were embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

RESULTS

Light microscopical observations showed that in all sporulation media vegetative cells increased in size after multiplication had ceased. Conjugation occurred between independent cells as well as between mother cell and bud. Prior to ascosporeogenesis often a large vacuole developed and many lipid droplets were observed in the cells. Asci contained 1–4 small, hat-shaped ascospores which were released from the ascus after spores had matured.

Electronmicroscopical observations

Cells, growing exponentially in media supplemented with glucose and ammoniumsulphate as the nitrogen source and in which the synthesis of alcohol oxidase is repressed, generally contained a single, small microbody with dimen-

Hansenula polymorpha cells were fixed/postfixed with KMnO_4 unless mentioned otherwise. m = mitochondrion; n = nucleus; p = peroxisome.

Fig. 1. Detail of a vegetative cell, grown in a medium with glucose and $(\text{NH}_4)_2\text{SO}_4$ from the mid-exponential growth phase, showing a typical so-called "glucose peroxisome" ($46\,500\times$).

Fig. 2. Detail of a vegetative cell, showing the large peroxisome present in the cells during exponential growth in media with glucose and urate as the nitrogen source ($48\,500\times$).

Fig. 3. Conjugation between two independent cells ($10\,000\times$).

Fig. 4. Detail of a young ascus developed in a medium with glucose and urate, showing a pro-spore wall (arrow) together with a small peroxisome in the epiplasm ($62\,500\times$).

Fig. 5. Survey of two spores developed in a medium with glucose and urate ($44\,500\times$).

Fig. 6. Detail of a vegetative cell, grown in the presence of glycerol, showing 3 peroxisomal profiles ($40000\times$).

Fig. 7. Detail of a spheroplast of a cell, grown in the presence of methanol, to illustrate the crystalline substructure of the peroxisomes, present in these cells ($31000\times$).

Fig. 8. Survey of cells, grown in the presence of methanol and urate, after growth had ceased. In most of the cells a large vacuole is present, together with lipid droplets and glycogen. In these cells peroxisomes are not visible. Compare the virtually unaffected cell below left ($34500\times$).

Fig. 9. Section through a cell, grown in a medium with methanol and urate, from the same growth phase as seen in Fig. 8, showing a number of small peroxisomes which remained after fragmentation of the organelles originally present. Compare Fig. 8 ($29000\times$).

Fig. 10. Young ascus, developed in a medium with methanol and urate, showing a spore initial with a still incomplete pro-spore wall. Beside the nucleus a peroxisome is present ($40000\times$).

sions of 0.1–0.2 μm (Fig. 1) (Veenhuis et al., 1979). However, when grown under the same conditions in the presence of urate as the nitrogen source, the cells generally contained a single microbody of 0.3–0.6 μm (Fig. 2). These organelles originated from the small microbodies, present in the inoculum cells, which had been grown on glucose and ammoniumsulphate (Veenhuis et al., 1979). The increase in dimension is associated with the synthesis of urate oxidase and catalase, which are present in these organelles when urate serves as the nitrogen source in the growth medium (Zwart, unpublished results). After diploidization and cessation of growth, generally a large vacuole was observed in the cells, together with glycogen and a number of lipid droplets (Figs. 3 and 8). At this stage of sexual reproduction the microbodies in the glucose – urate-grown cells decreased in size to 0.1–0.2 μm . The subsequent observations on the overall changes in cell ultrastructure during ascosporeogenesis in *H. polymorpha* were similar to those described for ascospore development in *H. wingei* (Black and Gorman, 1971). Thin sections of KMnO_4 -fixed ascospores, developed in repressed cells of *H. polymorpha*, revealed that in each spore generally 1–2 microbodies were present (Fig. 5). The organelles were already present in spore initials with an incomplete pro-spore wall and existed in close association with strands of ER. Development of spores was not synchronous in asci of *H. polymorpha*. In young asci, in which spore formation was still incomplete, microbodies were observed in the epiplasm (Fig. 4). However, when all spores were formed, epiplasmic microbodies were no longer detected. During subsequent maturation of spores we observed an increase in size of the microbodies in the spores. Mature spores contained microbodies of a round shape, with average dimensions of 0.2–0.3 μm , in close association with strands of ER (Fig. 5). Crystalline inclusions were not observed in these organelles.

Cells in which the synthesis of alcohol oxidase was derepressed (which is the case during growth in media with methanol, glycerol or dihydroxyacetone in the presence of urate as the nitrogen source), were characterized by a number of large microbodies in the cells. During growth in glycerol and dihydroxyacetone media up to 5 (Fig. 6) and during growth in methanol media up to 8 microbodies developed per cell. The organelles had a partly or completely crystalline substructure (Fig. 7), as was observed in thin sections of glutaraldehyde – $\text{OsO}_4/\text{K}_2\text{Cr}_2\text{O}_7$ fixed spheroplasts. After growth had ceased a large vacuole developed in the cells and the synthesis of glycogen and lipid droplets occurred (Fig. 8), similar to observations made in glucose-grown cells. In addition, the large microbodies, present in the cells, fragmented into a number of small, irregularly shaped organelles (Fig. 9). Subsequently, a number of these organelles degraded while generally 5–10 microbodies remained in the developing ascus. During spore initiation 1–3 of these microbodies were included into the developing ascospore (Fig. 10) together with other cell organelles such as the nucleus, mitochondrion and ER (Fig. 11). The microbodies existed in close association with strands of ER and had average dimensions of 0.4–0.5 μm (Fig.

12). They showed a crystalline substructure after glutaraldehyde – $\text{OsO}_4/\text{K}_2\text{Cr}_2\text{O}_7$ fixation. Crystalline microbodies of similar dimensions were also observed in ascospores obtained from malt agar plates (Fig. 13). As in glucose-grown cells development of spores was not synchronous in young asci of derepressed cells but when all spores were formed microbodies were no longer detected in the epiplasm.

Cytochemical staining experiments

The cytochemical staining experiments were performed in aerated incubation mixtures with diaminobenzidine (DAB) in the presence of a substrate for a specific oxidase in order to simultaneously demonstrate catalase and oxidase

Fig. 11. Section through a spore, developed in a medium with methanol and urate. Two large peroxisomes are visible within the spore (50 500 $\times$).

Fig. 12. Detail of a spore, developed in the presence of methanol and urate, demonstrating the close association between ER and peroxysome (arrow) (65 000 $\times$).

Fig. 13. Detail of a spore, developed on malt agar, showing the crystalline substructure of the peroxisomal matrix (62 500 $\times$).

Fig. 14. Section through a spore, developed in a medium with glucose and urate, showing a positively stained peroxisome after incubation with DAB and DL-alanine ($33\,500\times$).

Fig. 15. Section through a spore, developed in a medium with glucose and urate, incubated with DAB and methanol. The peroxisome remained unstained ($45\,500\times$).

Fig. 16. Section through a spore, developed in a medium with methanol and urate, showing a positively stained peroxisome after incubation with DAB and urate ($43\,000\times$).

Fig. 17. Stained peroxisome (see Fig. 16) with a crystalline substructure after incubation with DAB and methanol ($72\,500\times$).

Fig. 18. Section through a spore, developed in a medium with methanol and urate, after germination in the presence of glucose. In the original spore a typical "glucose peroxisome" is present (arrow) ($16\,000\times$).

Fig. 19. Section through a spore, developed in a medium with glucose and urate, after germination in the presence of methanol. Two large peroxisomes are visible in the original spore ($20\,500\times$).

activities (van Dijken et al., 1975; Veenhuis et al., 1978). In microbodies present in ascospores developed in cells grown on glucose-urate media, activity of catalase, amino acid oxidase and urate oxidase was demonstrated (Fig. 14); alcohol oxidase was not present (Fig. 15). The latter enzyme was invariably present in crystalline microbodies in ascospores, developed in derepressed cells, which had been grown on methanol, glycerol, dihydroxyacetone or malt extract (Fig. 17). Apart from alcohol oxidase, also amino acid oxidase and urate oxidase activity (Fig. 16) were demonstrated in these organelles. Control experiments, performed with DAB in the absence of an oxidase substrate or in the presence of aminotriazole to inhibit catalase activity, resulted in unstained microbodies. All incubations in the presence of DAB showed positively stained mitochondria in the ascospores. Since this staining was independent of any additional substrate, this reaction may be due to staining of mitochondrial peroxidases (Hoffman, Szabo and Avers, 1970). Cytochemical staining experiments for the demonstration of malate synthase activity (Trelease et al., 1974) gave negative results. Microbodies in both ascospores and vegetative cells remained unstained.

Germination experiments

Ascospores of *H. polymorpha*, grown on glucose or methanol, failed to germinate in tap water or in mineral media lacking a source of carbon or nitrogen, respectively. Germination of spores from glucose-grown cells in media supplemented with glucose or methanol started after a lag of about 3 hours. Similar results were obtained after inoculating spores from methanol-grown cells in the same media or spores from glucose-grown cells in glucose-containing media in the presence of methylamine as the nitrogen source. The phenomena observed during germination of spores of *H. polymorpha* were essentially similar to those described for the germination of ascospores of *H. holstii* (Das and Black, 1971). EM-observations of KMnO_4 -fixed cells showed that after germination in glucose-containing media of spores obtained from both repressed and derepressed cells, the first developing buds as well as the original spores contained microbodies (Fig. 18), typical for glucose-grown cells (Veenhuis et al., 1979). However, it remained unclear whether these organelles originated from the organelles present in the mature spores. Germination of spores from methanol-grown cells in methanol media resulted in an increase in size of the microbodies present in the spore. The organelles were also present in the buds which were the first to develop and originated from the microbodies present in the spore by division and subsequent migration to the bud. Growth of peroxisomes was also observed after germination of spores from glucose-grown cells in methanol media. The increase in size of the microbodies, present in these spores, started already during the lag phase. Germination of spores from glucose-grown cells in glucose media in the presence of methylamine as nitrogen source also resulted in growth and division of the microbodies present in the spore.

DISCUSSION

The occurrence and function of microbodies in vegetative haploid cells of the methylotrophic yeast *H. polymorpha* is characteristically associated with environmental conditions. During exponential growth in glucose-containing media, these cells contain a single small microbody of 0.1–0.2 μm (Veenhuis et al., 1979). Transfer of these cells into media supplemented with methanol as the carbon source results in the development of a number of large peroxisomes in these cells which contain large amounts of alcohol oxidase and catalase, key enzymes in methanol metabolism (van Dijken et al., 1976). These organelles have a partly or completely crystalline substructure, due to crystallization of alcohol oxidase molecules and originate from the small microbody, originally present in the glucose-grown cells by means of growth and division (Veenhuis et al., 1979). In vegetative cells the total number and volume of the microbodies is dependent on cultivation conditions. When *H. polymorpha* was grown in methanol-limited chemostats, up to 18 cubically shaped microbodies have been observed per cell, which together made up 80 % of the cytoplasmic volume (Veenhuis et al., 1978). During growth in media with glycerol or dihydroxyacetone in batch culture the synthesis of alcohol oxidase was partly derepressed and 3–5 microbodies were present making up about 15 % of the cytoplasmic volume (Egli et al., 1980; Veenhuis, unpublished results). Comparable observations have been made during growth of *H. polymorpha* in glucose-containing media when supplemented with different nitrogen sources. Growth in media with glucose and methylamine resulted in the development of large microbodies which contained amine oxidase and catalase, key enzymes in amine metabolism (Zwart et al., in press). Growth in the presence of urate or DL-alanine as nitrogen sources was associated with the synthesis of microbodies, containing besides catalase also urate oxidase and amino acid oxidase, respectively (Zwart, unpublished results). In all experiments described above the microbodies contained catalase together with at least one oxidase and may therefore be considered to be peroxisomes (De Duve and Baudhuin, 1966).

The present investigation has shown that peroxisomes are also present in ascospores of *H. polymorpha* under all sporulation conditions employed. So far peroxisomes have not been described in yeast ascospores, but the occurrence of microbodies in spores of filamentous fungi is well-known (Maxwell, Armentrout and Graves, 1977). In the latter organisms these organelles are possibly involved in the sporulation process. Hess and Weber (1974) have proposed that a peroxisome-based glyoxylate cycle might be important in sporulation, while microbodies present in zoospores of *Entophyctis* sp. and *Blastocladiella emersonii* isocitrate lyase and malate synthase have been found (Mills and Cantino, 1975) and it has been suggested that these organelles are involved in the metab-

olism of lipid reserves (Bimpong, 1975). Our experiments indicated that the peroxisomes, present in the cells of *H. polymorpha*, are probably not involved in ascosporeogenesis. This view is based on the following observations. Prior to spore initiation we invariably observed a drastic decrease in the total volume of peroxisomes in glucose-urate grown cells, as well as in methanol-, glycerol- and dihydroxyacetone-grown cells. Our results indicated that the peroxisomes in the spores which developed in these cells originated from the organelles, originally present in the vegetative cells. Cytochemical experiments showed that their enzymatic contents is a reflection of the enzymes present in peroxisomes in haploid cells, the latter being associated with the specific environmental conditions extant during growth of these cells. Therefore, a glyoxysomal function of the peroxisomes during spore development or spore germination is not expected. This is further supported by the results of the germination experiments which have shown that mature spores of *H. polymorpha* cannot use their endogenous reserves as a source of carbon and energy, or as a nitrogen source. Furthermore, in mature and germinating spores we have not observed a microbody-lipid globule complex, which is so apparent in zoospores of different fungi (Chong and Barr, 1973; Held, 1975; Powell, 1976) and is involved in β -oxidation of storage lipids during germination. In addition, cytochemical staining experiments to demonstrate malate synthase activity in peroxisomes in spores of *H. polymorpha* were negative.

However, the germination experiments clearly revealed that peroxisomes in spores are involved, as in haploid vegetative cells of *H. polymorpha*, in the oxidative metabolism of different sources of carbon and nitrogen. For instance, during the lag phase occurring when spores from glucose-grown cells germinated in methanol-containing media, we observed the conversion of the spore peroxisome into a large organelle with a crystalline matrix, probably due to the synthesis of alcohol oxidase. This process is similar to that described for transfer of glucose-grown cells into methanol-containing media (Veenhuis et al., 1979). Also germination of spores from glucose-grown cells in media with glucose and methylamine resulted in growth of the peroxisome originally present. This process is essentially similar to that described for transfer of cells grown on glucose-ammoniumsulphate into media containing methylamine as the nitrogen source (Zwart et al., in press). It may therefore be concluded that peroxisomes in germinating spores behave and function in a way similar to those present in vegetative cells and also that the enzymic profile of the organelles is identical to that of peroxisomes present in vegetative cells in which the spores have developed.

It was somewhat surprising to find that the lag observed during germination of spores with peroxisomes containing different enzymic profiles and placed under different environmental conditions was approximately constant. It must therefore be concluded that the presence of alcohol oxidase in spore peroxisomes is of no direct advantage to a spore germinating in an environment containing meth-

anol. In view of the finding that the enzymic profile of peroxisomes in germinating spores is rapidly adjusted to environmental conditions extant during germination it is suggested that peroxisomes in ascospores of *H. polymorpha* must be regarded as organelles that enable the cell to quickly match its metabolic machinery to the chemical composition of its environment after sexual reproduction.

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