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Synthesis and subcellular location of peroxisomal membrane proteins in a peroxisome-deficient mutant of the yeast *Hansenula polymorpha*

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We have studied the synthesis and subcellular location of peroxisomal membrane proteins (PMPs) in cells of a peroxisome-deficient (*per*) mutant of the methylotrophic yeast *Hansenula polymorpha*. Western blot analysis of methanol-induced cells of the *per* mutant, which had been growing in a continuous culture on a glucose/methanol mixture, indicated that various PMPs were normally synthesized. As in wild type (WT) cells, the levels of PMP synthesis appeared to be dependent on specific cultivation conditions, e.g. the carbon source used for growth. In contrast to WT controls, PMPs in methanol-induced *per* mutants were not subject to proteolytic degradation. Biochemical and immuno(cyto)chemical studies suggested that the PMPs in methanol-induced *per* cells were located in small proteinaceous aggregates, separated from peroxisomal matrix proteins that were also present in the cytosol. Vesicular membranous structures, resembling the morphology of intact peroxisomes, were never detected irrespective of the growth conditions employed.
Key words: *Hansenula polymorpha*/peroxisome-deficient mutant/peroxisomes/peroxisomal membrane/peroxisomal membrane proteins

Introduction

The proliferation and metabolic significance of peroxisomes in wild type (WT) cells of the yeast *Hansenula polymorpha* has been extensively studied (Veenhuis *et al.*, 1983a; Harder and Veenhuis, 1989). Massive peroxisome induction occurs during growth of cells on methanol as sole carbon source; in addition their synthesis is induced by different organic nitrogen sources (Zwart *et al.*, 1983a,b). Under these conditions the organelles play a key role in the growth of cells since they contain the enzymes involved in the primary oxidative metabolism of these growth substrates (Veenhuis and Harder, 1987; Harder and Veenhuis, 1989).

Recently, various peroxisome-deficient (*per*) mutants of *H.polymorpha* have been isolated (Titorenko *et al.*, 1993; Veenhuis, 1992; Waterham *et al.*, 1992). These mutants were identified within a collection of 260 methanol utilization (Mut^-) mutants previously described by Cregg *et al.* (1990). Physiological studies performed with these mutants indicated that the key enzymes of methanol metabolism are normally synthesized and active in the cytosol, indicating that intact peroxisomes are essential to support growth on methanol (van der Klei *et al.*, 1991). In contrast, the presence of a PER mutation does not hamper growth on organic nitrogen sources, the metabolism of which is mediated by peroxisomal enzymes in WT cells. Also these enzymes are present in the cytosol of *per* mutants and function with the same efficiency as in WT cells (Sulter *et al.*, 1990a). The phenotype of the *H.polymorpha per* mutants resembles that of human Zellweger cells, which are impaired in peroxisomal functioning (Schutgens *et al.*, 1986). Fibroblasts of Zellweger patients are characterized by the presence of different peroxisomal proteins in the cytosol (Wanders *et al.*, 1984; Santos *et al.*, 1985), which in part are unstable and subject to rapid degradation (Tager *et al.*, 1985; Schram *et al.*, 1986). It has long been anticipated that the primary defect in Zellweger cells is related to an incorrect assembly of the peroxisomal membrane. Recent studies, however, revealed that different peroxisomal membrane proteins are normally present (Santos *et al.*, 1988a; Wiemer *et al.*, 1989) and located in peroxisomal 'ghosts' (Santos *et al.*, 1988a,b; Wiemer *et al.*, 1989), which lack peroxisomal matrix enzymes. These results indicate that the Zellweger syndrome most probably is related to a dysfunctioning of the peroxisomal import machinery.

By analogy, peroxisomal membrane remnants may be present in yeast peroxisome-deficient mutants. This prompted us to study the presence and location of specific peroxisomal membrane proteins (PMPs) in a *per* mutant of the methylotrophic yeast *H.polymorpha* (*per* 7, 125-2E; Cregg *et al.*, 1990; Titorenko *et al.*, 1993). The results presented in this paper indicate that different PMPs of *H.polymorpha* (Sulter *et al.*, 1990b) are normally synthesized in the *per* mutant and located in the cytosol. As in WT cells, their levels of synthesis vary with growth conditions.

Results

Cell morphology

The overall morphology of fully derepressed cells of both the wild type (WT) and the peroxisome-deficient (*per*) mutant of *H.polymorpha* (identically grown in carbon-limited chemostat cultures on either glucose-choline or glucose-methanol mixtures) has been described before (van der Klei *et al.*, 1991). Cells of both strains are morphologically comparable in that they contain the usual cell organelles, but differ in that WT cells contain several large peroxisomes (Figure 1A), which are absent in the *per* mutant cells (Figure 1B). Instead, *per* cells contain a large cytosolic crystalloid, composed of alcohol oxidase (AO) protein (Figure 1B and C). The presence of peroxisomal membranes in the *per* mutant was investigated in both $KMnO_4$- and aldehyde/OsO_4-fixed cells. Extensive analysis of series of serial sections failed to resolve any vesicular or membranous structures akin to peroxisomal membranes in the *per* mutant, even when different growth conditions were

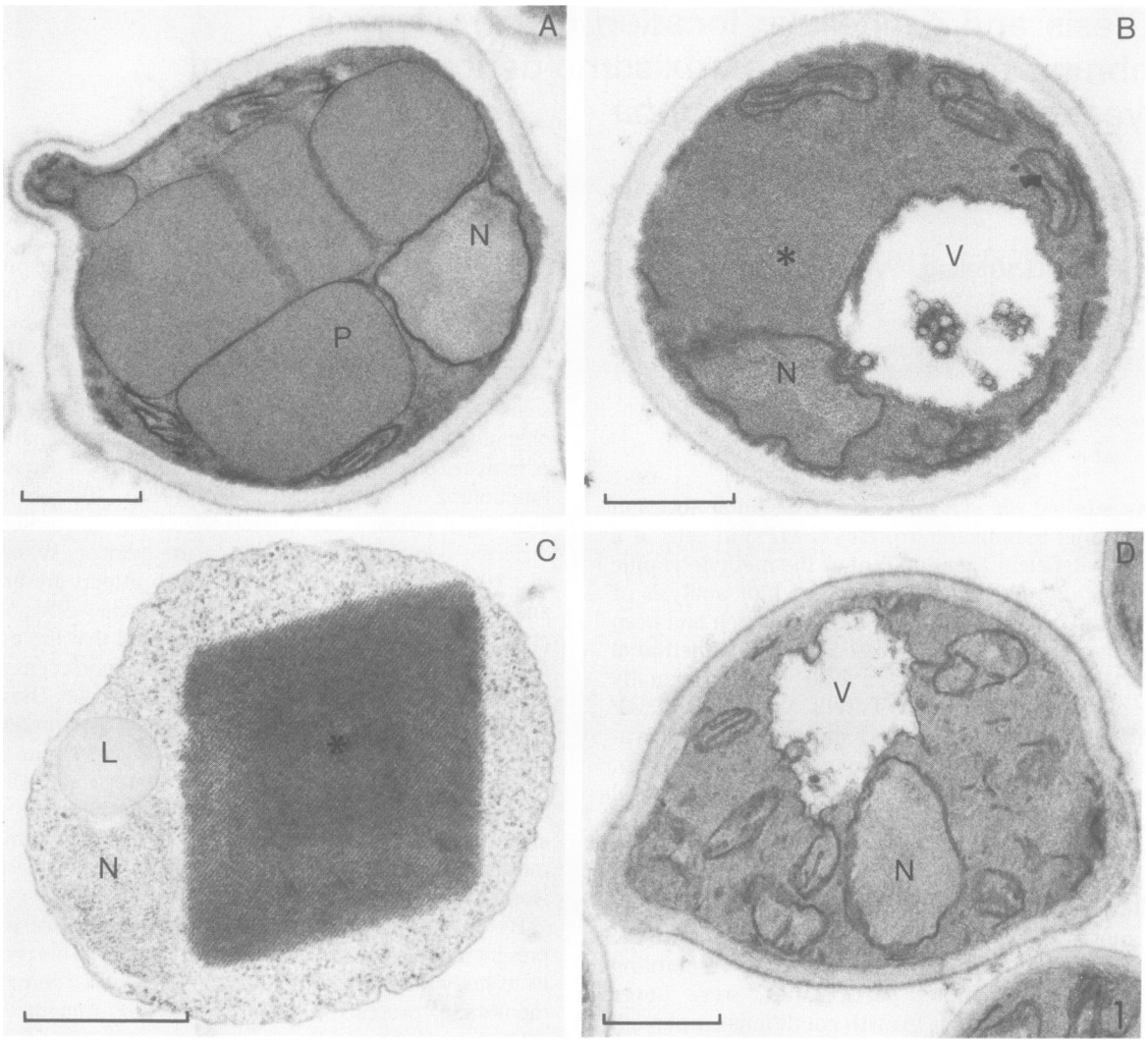

Fig. 1. Overall cell morphology of wild type (WT) and the peroxisome-deficient mutant (*per* 7, 125-2E) of *H.polymorpha*, grown in a carbon-limited chemostat on glucose/methanol. In the WT strain large cuboid peroxisomes are present (**A**). Intact peroxisomes are lacking in the *per* mutant (**B**). Also vesicular membranous structures resembling peroxisomes are absent. Instead, *per* mutants contain a large cytosolic crystalloid or alcohol oxidase (AO) (*) that is poorly preserved after $KMnO_4$ fixation (B), but readily resolved in glutaraldehyde-fixed spheroplasts (**C**). In glycerol-grown *per* cells, which lack AO crystalloids due to the relative low AO expression levels, peroxisomal remnants are also not observed (**D**). L, lipid droplet; N, nucleus; P, peroxisome; V, vacuole. The marker represents 0.5 μm.

employed (Figure 1B–D). Also in freeze–etch replicas, membrane fracture faces resembling the typical peroxisomal architecture (Sulter *et al.*, 1990b) were not detected (not shown).

Expression of peroxisomal membrane proteins

The presence of peroxisomal membrane proteins (PMPs) was studied in fully derepressed cells. Figure 2 shows a Western blot prepared from crude extracts of *per* mutants, incubated in batch cultures on methanol, decorated with polyclonal antibodies generated against purified peroxisomal membranes of WT *H.polymorpha* (Figure 2, lane 1). The overall pattern obtained is comparable to that seen in control experiments on identically, methanol-grown, WT cells (Figure 2, lane 2); however, the level of synthesis of individual PMPs (e.g. PMP31 and PMP51), recognized by these antibodies, may vary slightly between the two strains. Previous studies (Sulter *et al.*, 1990b) have shown that most of the PMPs in WT *H.polymorpha* are constitutively present, whereas others are

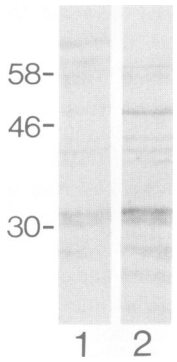

Fig. 2. Western blot, prepared from crude extract of derepressed WT and *per* mutant cells of *H.polymorpha*, using polyclonal antibodies raised against purified peroxisomal membrane proteins (PMPs). Lane 1, *per* mutant; lane 2, WT control cells. 15 μg of protein was loaded on each lane. PMPs decorated with specific polyclonal antibodies were detected by an enhanced light signal (see Materials and methods).

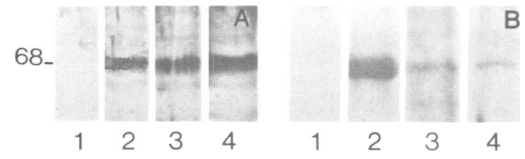

Fig. 3. Western blots showing the induction of PMP68 in *per* mutant (**A**) and WT control cells (**B**). Cells precultivated on glucose were transferred to fresh methanol-containing media; samples were taken at 0 (lanes 1) and 24 h (lane 2) of incubation in methanol. Lanes 3 and 4 show the fate of PMP68 after the shift of methanol-induced cells to glucose excess conditions; samples were taken at 2 (lane 3) and 4 h (lane 4) respectively. In lanes 1 and 2, 8 μg of protein were loaded; in lanes 3 and 4 protein concentrations were adapted to correct for growth. PMP68 decorated with specific polyclonal antibodies was detected by an enhanced light signal (see Materials and methods).

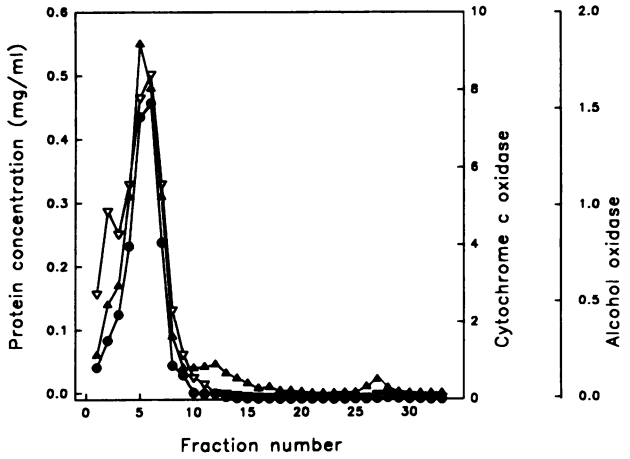

Fig. 4. Distribution of protein and specific activities of peroxisomal (alcohol oxidase) and mitochondrial (cytochrome *c* oxidase) marker enzymes after sucrose gradient centrifugation of a 30 000 *g* pellet fraction, obtained by differential centrifugation of homogenates of fully derepressed *per* cells, prepared by X-press treatment of intact cells. (▲) protein concentration; (▽) alcohol oxidase activity; (●) cytochrome *c* oxidase activity. Enzyme activities are expressed as μmol substrate consumed/min^{-1}/ml^{-1}.

inducible; the levels of synthesis of these latter PMPs are related to growth conditions. In order to establish whether this inducible nature is maintained in the *per* mutant, we studied the fate of a 68 kDa PMP (PMP68; Sulter *et al.*, 1990b) after a shift of cells from glucose to methanol. The results (Figure 3) indicate that induction of PMP68 is comparable in both the *per* mutant (Figure 3A, lanes 1 and 2) and control WT cells (Figure 3B, lanes 1 and 2). However, glucose-induced degradation, characteristic for WT *H.polymorpha* (Figure 3B, lanes 3 and 4), was not observed in the *per* mutant (Figure 3A, lanes 3 and 4).

Based on the assumption that PMP68—probably together with other PMPs—may be present in proteinaceous aggregates and/or peroxisomal membrane remnants (Santos *et al.*, 1988b), we attempted to enrich these structures by conventional fractionation methods that have been successfully applied for the isolation of intact peroxisomes (Douma *et al.*, 1987; Sulter *et al.* 1990a). As expected, after sucrose density centrifugation of the 30 000 *g* pellet obtained by differential centrifugation of homogenized spheroplasts of derepressed WT cells, mitochondria (located at 45% sucrose) and peroxisomes (located at 53% sucrose) were clearly separated (data not shown). When these procedures

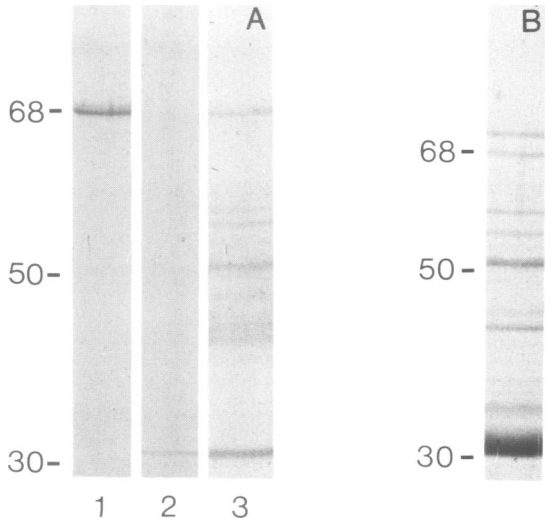

Fig. 5. A. Protein composition of the protein peak fraction located at 53% sucrose depicted in Figure 4. 1.0 μg of protein was electrophoresed on 12.5% SDS–PAGE, transferred to nitrocellulose and decorated with either specific antibodies against PMP68 (lane 1) or PMP31 (lane 2) or stained with silver (lane 3). **B**. shows the protein pattern of purified peroxisomal membranes from methanol-grown WT control cells (Sulter *et al.*, 1990b).

were performed with homogenates of derepressed *per* mutant cells, two protein peaks were obtained, one containing mainly mitochondria (at 45% sucrose) and a second minor peak (at 53% sucrose) enriched in PMP68 (data not shown). Because of the very low yield invariably obained by this procedure due to the fact that only part of the cells effectively lyse under these conditions, the experiment was repeated on homogenates obtained by X-press treatment of intact derepressed *per* mutants (from chemostat cultures on glucose/choline). Sucrose density centrifugation of the 30 000 *g* pellet of such homogenates again revealed two distinct protein peaks, located at 45% sucrose and 53.0% sucrose, respectively (Figure 4). This result was fully reproducible throughout a series of experiments and independent of the inducing compound (choline or methanol). Biochemical analysis of the different fractions obtained indicated that the first protein peak was highly enriched in AO and cytochrome *c* oxidase activities (Figure 4); electron microscopical analysis indicated that this peak consisted of AO crystalloids and mitochondria (not shown). However, this fraction fully lacked PMPs, as judged from Western blotting experiments. Analysis of the various fractions indicated that PMP68 and PMP31 (G.J.Sulter, K.Verheyden, G.Mannaerts, W.Harder and M.Veenhuis, submitted) specifically had sedimented in the 53% sucrose peak fraction (Figure 5A, lane 1 and 2, respectively). The protein pattern, obtained after SDS–PAGE of this peak fraction is shown in Figure 5A (lane 3). This pattern was comparable to the patterns obtained previously of purified peroxisomal membranes of methanol-grown *H.polymorpha* (Figure 5B; for detailed information on the protein composition of peroxisomal membranes, see Sulter *et al.*, 1990b). Distinct protein bands identical in both the PMP peak fraction and purified peroxisomal membranes were located at 31, 42, 51, 54, 57 and 68 kDa, respectively.

Subcellular location of peroxisomal membrane proteins

The subcellular location of PMPs was furthermore studied by immunochemical methods. For this purpose derepressed

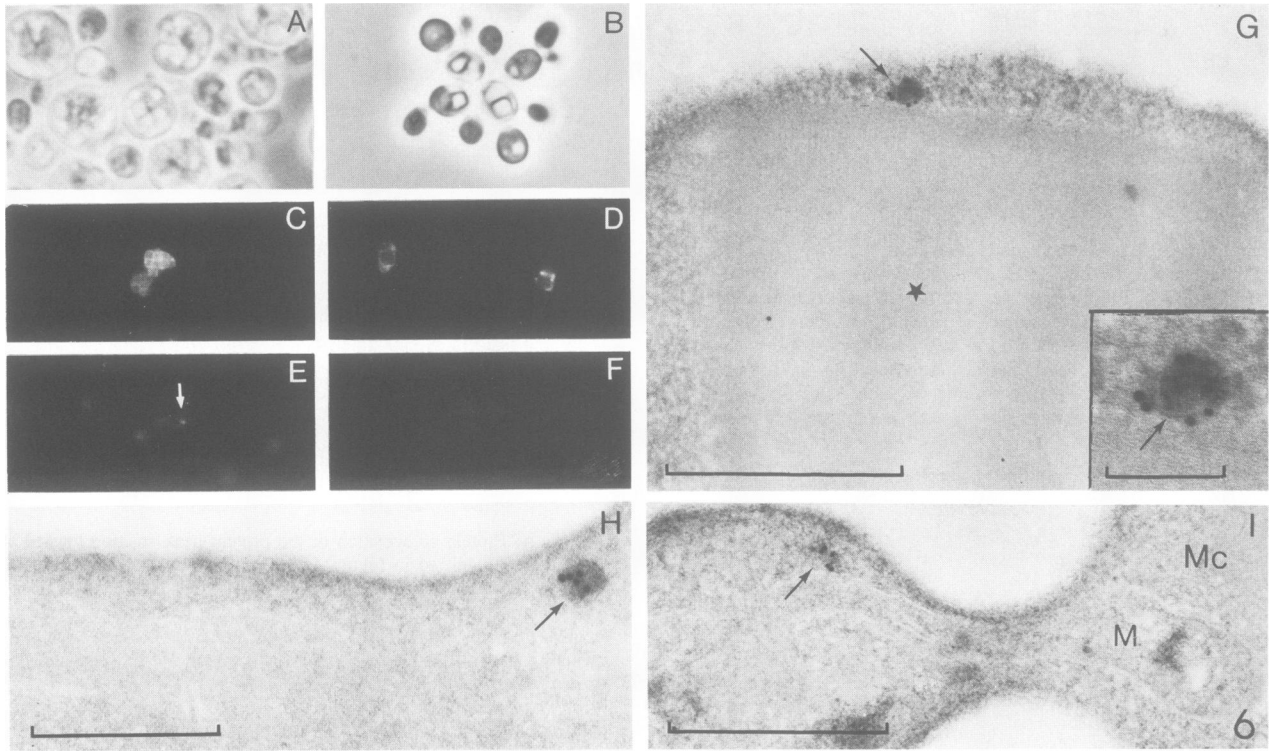

Fig. 6. Phase contrast light microscopy (A–B) and immunofluorescence experiments (C–F). Fully derepressed WT control cells (**A**) contain several cuboid peroxisomes, whereas *per* mutants are characterized by a large cubic inclusion (**B**). In immunofluorescent experiments using specific antibodies against purified peroxisomal membranes of *H.polymorpha* peroxisomal membranes in WT cells displayed strong fluorescence (**C**), whereas in *per* mutants one or few fluorescent spots were observed (**D**). In glucose-grown WT cells (**E**) invariably a single fluorescent spot (arrow) is observed that is never detected in glucose-grown *per* mutants (**F**). Immunocytochemical location of PMPs in fully derepressed *per* mutants. Typical labelling pattern of electron dense aggregates (arrow) observed in these cells after incubation of ultrathin sections of Lowicryl-embedded cells with protein A–gold and specific antibodies against PMP68 (**G**); the inset shows a high magnification, suggesting the association of membrane-like structures (arrow) with the labelled spot (bar represents 0.1 μm). Identical labelling patterns (arrow) were obtained when antibodies against purified peroxisomal membranes were used (**H**). A labelled aggregate (arrow; antibodies against purified peroxisomal membranes) present in a developing bud of *per* 7, grown in batch culture on glycerol (**I**). M, mitochondria, Mc, mother cell.

cells from both the WT and *per* mutant (grown under identical conditions in a chemostat on glucose/choline) were compared, since these cells contain either large cubic peroxisomes (WT strain) or a cytosolic AO crystalloid (*per* mutant), which both are readily recognized by light microscopy (Figure 6A and B).

By immunofluorescence and using antibodies against purified peroxisomal membranes of *H.polymorpha*, individual peroxisomes in WT cells displayed a strong fluorescence (Figure 6C); usually one (or infrequently very few) intensely fluorescent spots were observed in *per* mutants, often localized in close proximity to the large AO crystalloids (Figure 6D). Identical fluorescent patterns were obtained when specific antibodies against PMP68 of *H.polymorpha* were used. Both antisera could also resolve the small peroxisomes characteristic for WT cells grown on glucose (Figure 6E; Veenhuis *et al.*, 1979); however, a specific immunoresponse was not observed in glucose-grown *per* cells (Figure 6F).

At the subcellular level, using protein A–gold and antibodies against PMP68, specific labelling in fully derepressed *per* mutants was confined to small electron dense aggregates. In WT control cells solely the peroxisomal membrane was labelled (not shown). Typical examples of the aggregates are shown in Figure 6G. These electron dense spots may be associated with phospholipids, as was suggested from their ultrastructure at high magnifications (Figure 6G, inset). Double labelling experiments revealed that these spots did not contain matrix proteins (e.g. AO and dihydroxyacetone synthase, not shown). Identical labelling patterns were obtained when antibodies against purified peroxisomal membranes were used (Figure 6H). Since PMP68 is not recognized by the latter antiserum, these results indicate that the electron dense spots may represent protein–phospholipid aggregates containing different PMPs.

In partly derepressed *per* mutants (grown in batch cultures on glycerol) labelling of PMPs associated with distinct subcellular structures was not observed. Infrequently, few gold particles accumulated on very small spots of increased electron density, but we were unable to resolve the substructure (proteinaceous and/or membranous) of these spots (Figure 6I). Significant labelling in glucose-grown *per* mutants was never observed.

Discussion

This paper describes the synthesis and subcellular location of different peroxisomal membrane proteins (PMPs) in a peroxisome-deficient mutant (*per* 7, 125-2E; Cregg *et al.*, 1990; Titorenko *et al.*, 1993) of *H.polymorpha*. Our results indicate that in several aspects PMPs behave comparably in both wild type (WT) and *per* cells. First, the levels of synthesis and molecular masses were identical in both strains.

Secondly, in the *per* mutant, different PMPs are inducible as in WT cells. However, in *per* cells glucose-induced degradation of PMPs was not observed. In this respect the membrane proteins behaved like matrix proteins, which in *per* mutants of *H.polymorpha* also were not susceptible to carbon catabolite inactivation (van der Klei *et al.*, 1991). These results further strengthen the current view that the proteolytic turnover of both peroxisomal matrix and membrane proteins, as described for WT cells (Veenhuis *et al.*, 1983b), is dependent on the presence of intact organelles.

Unambiguous information on the subcellular location of PMPs in the *per* mutant was solely obtained in fully derepressed cells (grown in chemostat cultures on either glucose−choline or glucose−methanol mixtures). Under these conditions maximal levels of synthesis of both peroxisomal matrix (van der Klei *et al.*, 1991) and membrane proteins (Sulter *et al.*, 1990b) are obtained. Despite the fact that PMPs banded in sucrose gradients at the position of intact peroxisomes, membranous structures or vesicles resembling peroxisomal membranes were never observed in *per* mutants. It should be noted that peroxisomal membranes can be readily discriminated from other cellular membranes by morphological criteria (e.g. membrane width and architecture of fracture faces in freeze−etch replicas; Sulter *et al.*, 1990b). Instead, in *per* cells electron dense proteinaceous aggregates were observed which, based on their ultrastructure, may be associated with phospholipids (Figure 6G, inset). Since generally one single aggregate was found per cell, which was recognized by polyclonal antibodies against purified peroxisomal membranes as well as specific polyclonal antibodies against PMP68, we suggest that different PMPs are incorporated in one and the same aggregate. This implies that either these proteins have a distinct mutual affinity comparable to matrix proteins (van der Klei *et al.*, 1991, 1992) or alternatively that their sorting mechanisms are still functional and consequently, these aggregated structures may represent remnants of the peroxisomal membrane. However, these structures have lost the capacity to proliferate, which is a characteristic property of intact organelles in derepressed WT yeast cells (Veenhuis *et al.*, 1979; Kunau and Hartig, 1992). On the other hand, analysis of several series of serial sections indicated that aggregates may already be present in developing buds prior to closure of the cross wall between mother cell and bud (see Figure 6I). Therefore, maintenance of these structures, as is the case with small intact peroxisomes in fully repressed (glucose-grown) cells, may still be functional. Our failure to detect PMP aggregates in glucose-grown *per* mutants most probably is related to either the limiting resolving power of the ultrastructural methods used, which do not allow the visualization of very small aggregates or that at very low levels of synthesis aggregation does not occur. Summarizing, we have demonstrated that in a *per* mutant of *H.polymorpha* peroxisomal membrane proteins are induced as in WT cells and, at enhanced levels of synthesis, are located in protein−phospholipid aggregates. Based on their morphology, these aggregates cannot be referred to as 'ghosts', as described for human Zellweger cells (Santos *et al.*, 1988b; Wiemer *et al.*, 1989). The aggregate-like structures furthermore lack characteristic properties of intact yeast peroxisomes namely (i) the capacity to proliferate and (ii) the susceptibilty to carbon catabolite inactivation.

Materials and methods

Microorganisms and growth conditions

Both wild type (WT) *H.polymorpha* de Morais et Maya CBS 4732 and a peroxisome-deficient (*per*) mutant derived from this strain (*per* 7, 125-2E) (Cregg *et al.*, 1990; Titorenko *et al.* 1993), were incubated in batch cultures at 37°C in the mineral medium described by van Dijken *et al.* (1976), supplemented with either 0.5% (w/v) glucose, 0.5% (v/v) glycerol or 0.5% (v/v) methanol as carbon source.

Both strains were also grown in carbon-limited chemostat cultures at 37°C at a dilution rate (D) of 0.08 h in the mineral medium described previously by Douma *et al.* (1985). Cells were grown on either 0.25% (w/v) glucose as carbon source in the presence of choline (0.25% w/v) as nitrogen source or on a mixture of 0.25% (w/v) glucose and 0.1% (v/v) methanol.

Induction experiments

The induction of peroxisomal membrane proteins was studied after a shift of fully repressed, glucose-grown cells (Veenhuis *et al.*, 1979) to methanol excess conditions. For this purpose cells of both the WT and the *per* mutant were precultured in batch cultures on 0.5% glucose as carbon source. In the late exponential growth phase (A_{663} = 2.5) cells were diluted five times in fresh mineral medium containing 0.5% methanol. Samples were taken after 0 and 24 h incubation.

Since peroxisomal proteins in WT *H.polymorpha* are sensitive to carbon catabolite inactivation (Veenhuis *et al.*, 1983b), we studied whether this mode of regulation also occurred in the *per* mutant. Cells, incubated for 24 h in methanol-containing media (as described above) were diluted 5-fold into fresh glucose-containing (0.5% w/v) media and incubated at 37°C. Samples were taken at 2 and 4 h after the shift.

Enzyme assays

Crude extracts were prepared as described before by van der Klei *et al.* (1991). All enzyme assays were performed at 37°C. Alcohol oxidase was assayed according to Verduyn *et al.* (1984), cytochrome *c* oxidase as described by Douma *et al.* (1985). Enzyme activities are expressed as μmol substrate consumed or product formed min/mg protein. Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as standard.

The polypeptide composition of crude extracts was studied by SDS−PAGE (Laemmli, 1970), stained with silver. Western blotting was carried out according to Kyhse-Andersen (1984), using a semi-dry electroblotter (Ancos, Denmark); proteins were visualized by either alkaline phosphatase-conjugated secondary antibodies or on film by light emission created by the presence of ECL Western blotting detection system (Amersham).

Polyclonal antibodies raised against purified peroxisomal membranes, a specific 68 kDa PMP (PMP68) and a 31 kDa PMP (PMP31) of *H.polymorpha* were used (Sulter *et al.*, 1990b).

Cell fractionation

Conventional cell fractionation experiments including differential and sucrose density centrifugation of homogenized spheroplasts (Douma *et al.*, 1987) were performed on cells of the *per* mutant and WT strain of *H.polymorpha* grown in chemostat cultures on a glucose−choline mixture. In addition, intact *per* cells were fractured by X-press (Biotech, Sweden) treatment. Prior to fracturing, cells were washed with 50 mM potassium phosphate buffer (pH 7.5) and resuspended in the same buffer (1 g wet weight/ml). The resulting homogenate was subjected to differential centrifugation with the following centrifugation steps: 10 min at 600 *g*; 10 min at 12 000 *g* and 30 min at 30 000 *g*. The 30 000 *g* pellet was layered on top of a discontinuous sucrose gradient and centrifuged in a Sorvall SS90 vertical rotor at 34 500 *g* for 3 h at 4°C (Sulter *et al.*, 1990b).

Immunofluorescence

Intact cells were washed once in distilled water, resuspended in 40 mM potassium phosphate buffer pH 6.5, supplemented with 3% (v/v) formaldehyde and prefixed for 2 h at room temperature. The fixed cells were incubated in 2.0 M sorbitol, 50 mM DTT and 25 mM EDTA (freshly prepared) for 15 min, washed twice in 2.0 M sorbitol solution and subsequently incubated in the presence of Zymolyase 20T (16.6 mg/g of cells, wet weight) in 0.1 M sodium citrate buffer (pH 5.5) supplemented with 2.0 M sorbitol and 5 mM EDTA at 30°C for 90 min for spheroplast formation. Next, the spheroplasts were washed once in 2.0 M sorbitol and suspended in a 1:1:1 mixture of 1% BSA, 2.0 M sorbitol and phosphate buffered saline (buffer A). Aliquots of these samples (usually 200 μl) were incubated with antisera for 16 h at room temperature, washed three times with buffer A and subsequently incubated with 200 μl buffer A supplemented with 2 μl anti-rabbit fluorescein isothiocyanate (FITC) for 1 h. After washing

three times in buffer A, the cells were examined in a Zeiss Axioskop epifluorescence microscope equipped with a blue excitation bandpass filter (BP 450–490 nm) and an emission cut-off at 520 nm.

Electron microscopy

Intact cells were fixed in 1.5% (v/v) $KMnO_4$ for 20 min. Spheroplasts and subcellular fractions were fixed in 6% (v/v) glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.2) for 60 min at 0°C, followed by a post-fixation in a 1:1 mixture of 0.5% (w/v) OsO_4 and 2.5% (w/v) $K_2Cr_2O_7$ 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 Philips EM 300 or a Philips CM 10. Intact cells were prepared for freeze–etching as described by Veenhuis *et al.* (1979).

Immunocytochemistry

Intact cells were fixed in 3% (v/v) glutaraldehyde in 100 mM sodium cacodylate buffer (pH 7.2) for 60 min at 0°C, dehydrated in a graded ethanol series and embedded in Lowicryl K_4M. Immunolabelling was performed on ultrathin sections using specific antibodies against alcohol oxidase, purified peroxisomal membranes and PMP68 by the protein A–gold method described by Slot and Geuze (1984).

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