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Cold-inducible selective degradation of peroxisomes in *Hansenula polymorpha*

Janet A. Komduur, Anna Rita Bellu, Kevin Knoops,
Ida J. van der Klei, Marten Veenhuis *

*Laboratory of Eukaryotic Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen,
PO Box 14, 9750 AA Haren, The Netherlands*

Abstract

Exposure of *Hansenula polymorpha* cells, grown in batch cultures on methanol at 37 °C, to a cold treatment (18 °C) is paralleled by a rapid degradation of peroxisomes present in these cells. Remarkably, the events accompanying organelle degradation at 18 °C are similar to those of selective glucose-induced peroxisome degradation in wild-type cells, described before. This observation was strengthened by the finding that cold-induced peroxisome degradation was not observed in mutants impaired in selective peroxisome degradation (Atg⁻ mutants). Biochemical data indicated that the onset of peroxisome degradation was not triggered by the inactivation of peroxisome function due to the fall in temperature. We show that our findings have implications in case of fluorescence microscopy studies that are generally not conducted at physiological temperatures and thus may lead to strong morphological alterations unless proper precautions are taken.

1. Introduction

Peroxisomes belong to the class of so-called microbodies and are characterized by the presence of one or more hydrogen peroxide-producing oxidases, together with catalase. The variety in their metabolic functions is unprecedented and is dependent on the organism in which they occur and sometimes even on the developmental stage of the organism [1–3]. In fungi, also including yeast, these organelles are generally involved in the primary metabolism of a number of unusual compounds used for growth, e.g. oleic acid, C1-compounds like methanol or methylamines, purines and D-amino acids [4,5]. However, also biosynthetic functions have been described; peroxisomes play a role in the synthesis of specific amino acids [6,7] and the production of β-lactam antibiotics by fungi [8].

Generally, in fungi microbody synthesis is induced during adaptation of cells to a new environment, in which one or more peroxisomal functions are required for growth [9]. Highest induction levels are observed in methylotrophic yeast species, in which peroxisomes may occupy up to 80% of the total cytoplasmic volume[10]. Remarkably, the opposite process, selective peroxisome degradation (also termed macropexophagy), is initiated when induced cells are placed in fresh environments in which the organelles are redundant for growth [11].

* Corresponding author. Tel.: +31 50 363 2176; fax: +31 50 363 8280.
E-mail address: m.veenhuis@biol.rug.nl (M. Veenhuis).

Peroxisomes do not contain DNA or a protein-synthesizing machinery. Hence, peroxisomal matrix proteins are encoded by nuclear genes. The analysis of various *PEX* genes that are crucial for peroxisome biogenesis has revealed much of the mechanisms of matrix protein sorting, but still has failed to resolve the details of the translocation step of newly synthesized proteins across the peroxisomal membrane. Since a distinct translocon, as e.g. observed for mitochondria and endoplasmatic reticulum (ER), was not detectable, alternative mechanisms have been proposed (reviewed by [12]). Some of these models predict that peroxisome formation involves the fusion of membrane vesicles. Titorenko et al. [13] have proposed that, in *Yarrowia lipolytica* wild-type cells, upon induction of peroxisome biogenesis, peroxisomes develop by fusion of ER-derived pre-peroxisomal vesicles that contain different sets of matrix proteins [14]. Also Faber et al. [15] have proposed a role for vesicle fusion in peroxisome biogenesis in *Pichia pastoris*.

In general, vesicle fusion processes are temperature-dependent and can be slowed down by decreasing the temperature. This prompted us to investigate whether in *Hansenula polymorpha* hypothetically existing pre-peroxisomal vesicles would accumulate upon a sudden decrease of the growth temperature at conditions of optimal peroxisome proliferation.

Unexpectedly, we observed that under these conditions in fact the opposite of peroxisome development occurred, namely a rapid selective degradation of the organelles. The mode of degradation was identical to the selective degradation process, induced after a shift of cells from methanol to glucose. Accumulation of pre-peroxisomal vesicles was not observed. The details of the cold shock-induced macropexophagy are included in this paper.

2. Materials and methods

2.1. Micro-organisms and growth conditions

Wild-type *H. polymorpha* CBS4732 and the *H. polymorpha* deletion strain *atg1* that is affected in selective peroxisome degradation [16], were used in this study. The cells were grown in batch cultures at 37 °C in mineral media (MM) [17] supplemented with glucose (0.5% w/v) or methanol (0.5% v/v) as respective carbon sources. For cold induction experiments cells were grown to the mid-exponential growth phase on methanol (OD$_{660}$ = 2.0–2.3) at 37 °C. Subsequently the cultures were rapidly cooled to 18 °C and further incubated at the low temperature. Samples were taken at regular intervals after the temperature shift for further analysis.

2.2. Biochemical methods

Alcohol oxidase (AO) activity was assayed as described before [18] using crude *H. polymorpha* cell extracts [19]. Cytochrome *c* oxidase (CCO) activity was determined by established procedures [20]. Formaldehyde and formate dehydrogenase activities were measured as described by Van Dijken et al. [21]. Determination of protein concentration, SDS–PAGE and Western blotting were performed by established procedures.

2.3. Electron microscopy

Cells were fixed and prepared for electron microscopy as described previously [19]. Immunolabeling was performed on ultrathin sections of Unicryl-embedded cells, using specific antibodies against *H. polymorpha* AO and gold-conjugated goat anti-rabbit antibodies [19].

3. Results

The cold exposure experiments were performed on cells, grown in batch cultures at 37 °C on methanol as sole carbon source to the mid-exponential growth phase, OD$_{663}$ ∼ 2.0. After an abrupt temperature shift of the culture down to 18 °C, cultivation was continued at
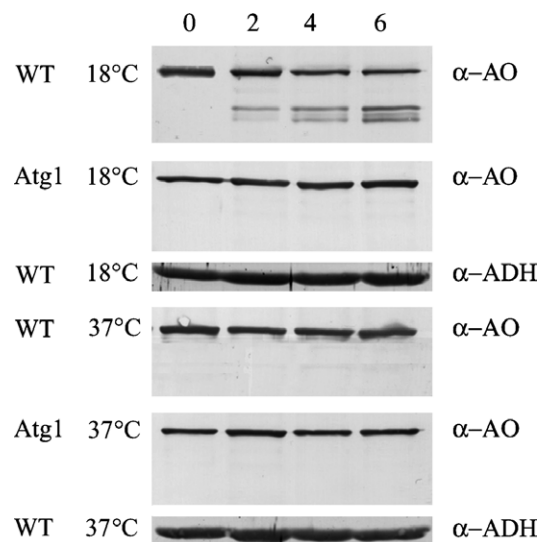
Fig. 1. Western blots showing the patterns of peroxisomal AO protein and cytosolic ADH, after exposure of wild-type (WT) and *atg1* cells, grown at 37 °C, to a cold treatment. Samples were taken at the onset of the experiment ($t = 0$) and after 2, 4 and 6 h of incubation of cells at 18 °C. Equal volumes of the cultures were loaded per lane. The blots were decorated with α-AO or α-ADH antibodies, respectively. The data show that the reduction of AO protein at 18 °C is specific and did not occur for ADH protein. The reduction of AO protein did not occur in WT cells that were kept at 37 °C or in *atg1* cells that are impaired in selective peroxisome degradation.

18 °C and samples were taken at regular time intervals for biochemical and structural analysis.

Western blots, prepared from crude extracts of cells harvested 0, 1, 2 or 4 h after the shift of cells to 18 °C, revealed that the levels of AO protein decreased during incubation of cells at decreased temperature (Fig. 1). Similar blots decorated with antisera against the cytosolic enzyme alcohol dehydrogenase (ADH) indicated that ADH levels stayed equal over the time span of the experiment. Control cells that were kept at 37 °C showed no reduction in AO or ADH protein levels. Together these data indicate that peroxisomes were not degraded by non-selective autophagy.

The kinetics of AO reduction at 18 °C were comparable to those observed in cells exposed to excess glucose at 37 °C to induce selective peroxisome degradation (data not shown). We also analyzed the fate of mitochondrial CCO after the shift of cells to 18 °C. The data indicated that CCO activities decreased in the 4-h time interval to approximately 80% of the original activities present in the cells, grown at 37 °C prior to the shift.

Ultrastructural studies were performed to analyze the morphological events accompanying the reduction in AO protein. At the onset of the cold shift ($t = 0$) the cells showed the usual peroxisome morphology and contained several peroxisomes characterized by the presence of AO protein, while this protein was absent in vacuoles (Fig. 2(a)). Already within 30 min after the shift to 18 °C peroxisome sequestration was generally detectable. Sequestering membranes are visualized as strong electron-dense layers that closely surround the organelle (Fig. 2(b)). One hour after the shift, AO protein was observed in the vacuoles of the bulk of the cells (Fig. 2(c)), but not in those of WT controls kept at the normal physiological temperature (data not shown). Together these data suggest that the cold shock has induced macropexophagy. Four hours after the shift to 18 °C only few small peroxisomes were left in the cells (not shown).

Since the morphological events accompanying cold shock-induced peroxisome degradation were identical to those of glucose-induced macropexophagy, we also
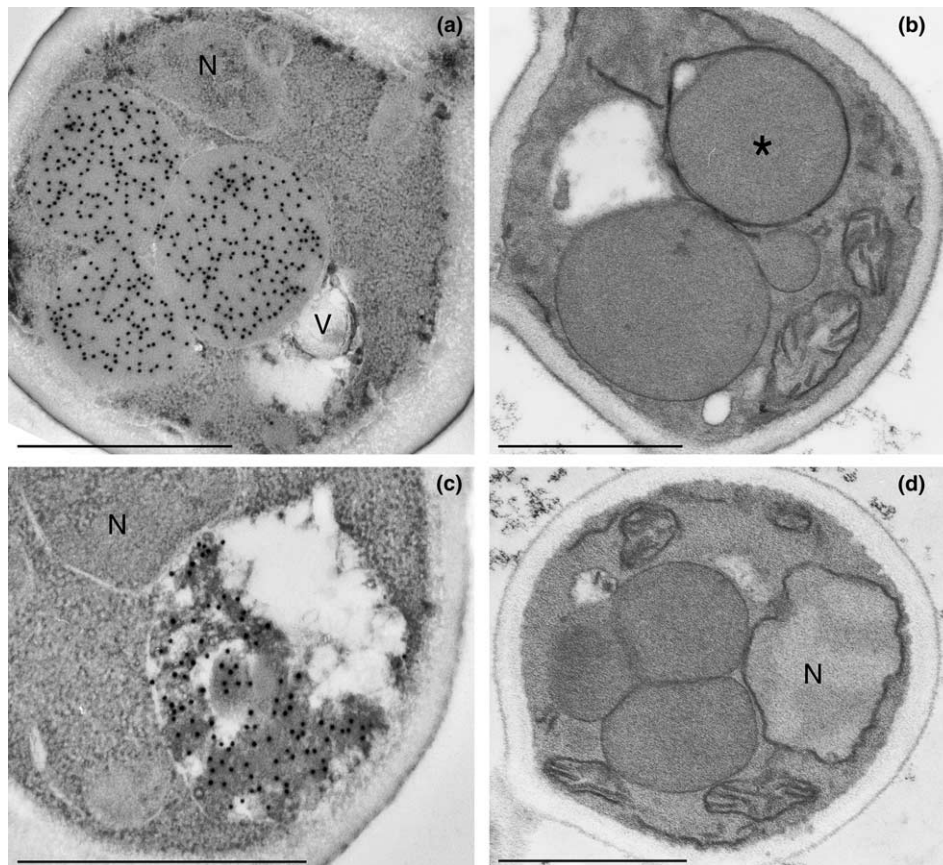

Fig. 2. Electron micrograph of a wild-type cell, pre-grown at 37 °C on methanol to $OD_{663} \sim 0.8$, to show the normal peroxisome profiles that are labeled in immunocytochemical experiments, using α-AO antibodies (a). Thirty minutes after the shift of cells to 18 °C, sequestration of individual peroxisomes could be observed as strong electron-dense layers that closely surround the organelle (b). After one hour of incubation peroxisome degradation was evident by the presence of AO protein (labeled with α-AO antibodies) in the vacuole (c). In *atg1* control cells the number of peroxisomes remained constant upon incubation of cells at 18 °C (d) and degradation events were not observed. N, nucleus; V, vacuole. The bar represents 0.5 μm.
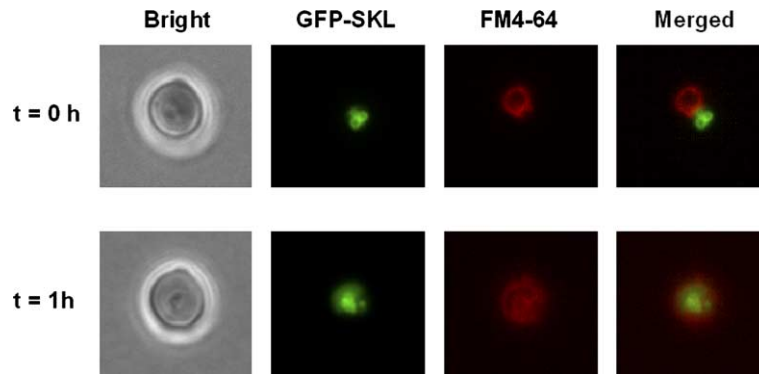
Fig. 3. Confocal fluorescence microscopy experiment, in which wild-type GFP-SKL-producing cells, grown on methanol at 37 °C, were transferred to a microscope slide and observed in the confocal laser scanning microscope for 1 h at room temperature (approximately 20 °C). Vacuoles are stained with the vacuole tracker FM4-64 (Molecular Probes, Eugene, OR, USA). The data show that the cells at the onset of the experiment ($t = 0$) contain three peroxisomes, two of which are incorporated in the vacuole after 1 h of observation. Note that the vacuole has increased in size in this time span.

analyzed the fate of peroxisomes in mutant cells that are impaired in macropexophagy (Atg⁻ mutants). Exposure of methanol-grown *atg1* cells, shifted from 37 to 18 °C, was not associated with a strong decrease of AO (Fig. 1) which remained at the levels observed in control cells at 37 °C. Also electron-microscopically no characteristics of macropexophagy were detected after the transfer of *atg1* cells to the lower temperature. Four hours after the shift the cells still contained normal peroxisome numbers (Fig. 2(d)).

A possible explanation for the observed cold shock-induced peroxisome degradation could be related to the inactivation of peroxisome functions, e.g. caused by the accumulation of formaldehyde produced from methanol. Enzyme measurements in crude extracts, prepared from methanol-grown wild-type *H. polymorpha* cells revealed that the specific activities of AO, formaldehyde dehydrogenase and formate dehydrogenase were not strongly reduced when measured at 18 °C, and amounted to approximately 80% of the values obtained at 37 °C (data not shown). Therefore, methanol catabolism can normally proceed at 18 °C and accumulation of toxic intermediates (formaldehyde, formate) is unlikely.

Remarkably, peroxisome degradation was also initiated in methanol-grown wild-type *H. polymorpha* cells that were analyzed in the confocal laser scanning microscope at room temperature (approximately 20 °C) for periods up to 60 min. It this time span most of the peroxisomes that were present in the cells were gradually incorporated in the vacuole (Fig. 3).

4. Discussion

We demonstrated that an abrupt shift of methanol-grown cells of *H. polymorpha* from the physiological growth temperature (37 °C) to lower temperatures (18 °C) induces selective degradation of peroxisomes.

This unexpected phenomenon strongly resembled glucose-induced selective peroxisome degradation (macropexophagy), regarding both the morphology and the biochemistry of the process. As in macropexophagy, cold treatment-induced peroxisome degradation is selective; mitochondrial or cytosolic marker enzymes are not drastically altered. That the organelles have indeed been subjected to macropexophagy is underscored by the finding that peroxisome degradation did not occur in a constructed mutant that is impaired in macropexophagy [22].

It remained unclear what the advantage is for the cell to remove peroxisomal activities upon a shift of cells from 37 to 18 °C. In general, many cellular processes are affected during cold acclimation (e.g., enzyme activities, membrane fluidity, cytoskeleton organization), so possible causes for the macropexophagy effect are numerous. One possible explanation as to why peroxisomes are degraded upon a temperature downshift is related to a possible inactivation of peroxisome function, for instance related to the accumulation of toxic compounds (e.g., formaldehyde or formate) generated from methanol. As shown before [23], *H. polymorpha* cells actively degrade chemically-inactivated peroxisomes. However, the specific activities of the enzymes of methanol catabolism (AO, formaldehyde and formate dehydrogenase) were not strongly reduced at 18 °C, rendering this scenario highly unlikely.

It is also possible that in *H. polymorpha*, as in plants and bacteria, osmolytes such as sugars (e.g., trehalose) accumulate during cold stress [24,25]. The precise mode of action of these compounds is unknown, but it is suggested that they may act as chemical chaperones by stabilizing native states of proteins or by acting as radical scavengers. Also, cells may react to cold stress with the production of glycerol [26]. Glycerol or sugars or their derivatives might induce macropexophagy in *H. polymorpha*, exposed to cold. However,

the possible role of such compounds requires further investigation.

A main implication of our data is related to structural studies of yeast peroxisomes, both by electron and fluorescence microscopy. Generally, such experiments involve temporally handling of the cells at room temperature, and thus below the physiological temperature. Our confocal laser scanning microscopy studies on methanol-grown *H. polymorpha* cells, producing the fusion protein GFP-SKL, have shown that peroxisome degradation may indeed be initiated after prolonged inspection of cells at room temperature. Therefore, care must be taken in such experiments either by observing cells for rather short periods or by using a temperature control unit.

Acknowledgements

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