Divergent modes of autophagy in the methylotrophic yeast *Pichia pastoris*

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SUMMARY

The budding yeast *Pichia pastoris* responds to methanolic media by synthesizing high levels of cytosolic enzymes (e.g. formate dehydrogenase) and peroxisomal enzymes (e.g. alcohol oxidase), which are necessary to assimilate this carbon source. Major alterations in cellular metabolism are initiated upon a shift in carbon source to ethanol or glucose. These alterations require the synthesis of new proteins and the rapid degradation of those enzymes no longer needed for methanol utilization. In this study, we have measured cytosolic and peroxisomal enzyme activities and examined the fate of morphologically distinct peroxisomes to assess the degradative response of this yeast during nutrient adaptation. Utilizing biochemical, morphological and genetic approaches, we have shown that there exist in *P. pastoris* at least two pathways for the sequestration of peroxisomes into the vacuole for degradation. The ethanol-induced pathway is independent of protein synthesis and includes an intermediate stage in which individual peroxisomes are sequestered into autophagosomes by wrapping membranes, which then fuse with the vacuole. This process is analogous to macroautophagy. The glucose-induced pathway invokes the engulfment of clusters of peroxisomes by finger-like protrusions of the vacuole by a process analogous to microautophagy.

Unlike ethanol adaptation, glucose stimulated the degradation of formate dehydrogenase as well. Peroxisomes remained outside the vacuoles of glucose-adapted cycloheximide-treated normal cells, suggesting that protein synthesis is required for peroxisome entry into the yeast vacuole. Two complementary mutants (gsa1 and gsa2) that are unable to degrade peroxisomes or formate dehydrogenase during glucose adaptation were isolated. The mutated gene products appear to function in one or more events upstream of degradation within the vacuole, since ethanol-induced peroxisome degradation proceeded normally in these mutants and peroxisomes were found outside the vacuoles of glucose-adapted gsa2 cells. Mutants lacking vacuolar proteases A and B were unable to degrade alcohol oxidase or formate dehydrogenase during ethanol or glucose adaptation. Peroxisomes were found to accumulate within the vacuoles of these protease mutants during adaptation. Combined, the results suggest that there exist in *Pichia pastoris* two independent pathways for the sequestration of peroxisomes into the vacuole, the site of degradation.

Key words: autophagy mutants, macroautophagy, microautophagy, peroxisomes, *Pichia pastoris*, proteasomes, vacuole

INTRODUCTION

Yeast cells are capable of rapidly responding to changing environmental conditions by selectively regulating the quantities and identities of endogenous proteins and organelles. This control is modulated by altering protein synthesis and/or degradation. Metabolite regulation of gene expression in yeast has been the subject of many ongoing studies and recent reviews (Trumbly, 1992). However, changes in protein degradation in response to nutrient changes have not yet been clearly defined. (Trumbly, 1992). Changes in protein degradation have been the subject of many ongoing studies and recent reviews. Metabolite regulation of gene expression in yeast has control is modulated by altering protein synthesis and/or degradation. The glucose-induced pathway invokes the engulfment of clusters of peroxisomes by finger-like protrusions of the vacuole by a process analogous to microautophagy.
mechanism of delivery of proteins and organelles to the vacuole remain largely unexplored. The requirements for uptake and degradation of the cytosolic protein fructose-1,6-bisphosphatase have been investigated in this yeast. Holzer and Purwin (1986) provided evidence suggesting that phosphorylation of this protein is necessary to target it for vacuolar degradation.

Several investigators have made use of mutant strains in which the genes for one or both PrA and PrB have been disrupted. Chiang and Schekman (1991) have used such mutants to show that fructose-1,6-bisphosphatase is degraded in the vacuole upon glucose adaptation. In addition, since metabolite-induced degradation of fructose-1,6-bisphosphatase requires protein synthesis and is not observed in some sec mutants, they have proposed that a membrane protein must be synthesized for transport of fructose-1,6-bisphosphatase into the vacuole for degradation. Ribosomes, mitochondria and membrane vesicles can be seen in the yeast vacuole of proteinase mutants that have been starved for nitrogen. It appears that entry of these organelles into the vacuole is enhanced by nitrogen starvation and proceeds by a process analogous to macroautophagy as defined for mammalian cells (Baba et al., 1994).

The methylotrophic yeasts, e.g. *Pichia pastoris*, *Hansenula polymorpha* and *Candida boidinii*, readily grow in media containing methanol as the sole carbon and energy source. During methanolic growth these yeasts synthesize the high levels of peroxisomal and cytosolic enzymes that are necessary for the utilization of methanol (Egli et al., 1980; Fukui et al., 1975a,b). Clusters of large peroxisomes that can be easily identified morphologically often occupy ≥250% of total cellular volume. Upon adaptation to an alternative carbon source (i.e. glucose or ethanol), the peroxisomes are rapidly degraded in the vacuole. In *H. polymorpha*, peroxisomes are sequestered within a wrapping membrane of unknown origin during glucose adaptation. This autophagosome then fuses with the yeast vacuole wrapping membrane of unknown origin during glucose adaptation to an alternative carbon source (i.e. glucose or ethanol). This autophagosome then fuses with the yeast vacuole wrapping membrane of unknown origin during glucose adaptation to an alternative carbon source (i.e. glucose or ethanol). This autophagosome then fuses with the yeast vacuole wrapping membrane of unknown origin during glucose adaptation to an alternative carbon source (i.e. glucose or ethanol).

PrA and PrB.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions**

GS115, a histidine auxotroph of an essentially wild-type *P. pastoris* strain (Cregg et al., 1985) was a generous gift from J. M. Cregg (Oregon Graduate Institute, Beaverton, OR). Preculture medium was YPD, consisting of 1% yeast extract (Difco Laboratories, Detroit, MI), 2% Bacto-peptone (Difco), and 2% dextrose. Methanol induction medium consisted of 6.7 g/l yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI), 40 mg/l L-histidine, 40 µg/ml biotin, and 0.5% methanol. To produce peroxisome repression medium absolute ethanol was added to a final concentration of 0.5% or solid glucose was added to a final concentration of 2% to methanol-induction cultures in stationary phase. Nitrogen deprivation medium was composed of 1.7 g/l yeast nitrogen base without amino acids and ammonium sulfate (Difco), 40 mg/l L-histidine, 40 µg/ml biotin, and 2% glucose. *P. pastoris* was grown at 30°C with 250 rpm shaking for all experiments. All chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA) except as noted.

**Mutant strains of *P. pastoris***

A wild-type strain of *P. pastoris* (GS115) was mutagenized according to the method of Cregg et al. (1990) to produce ~95% killing and frozen for later screening. Mutants were identified by direct colony assay. Samples of mutagenized cells were diluted appropriately to yield 500 cells per plate when spread on methanol induction plates (6.7 g/l yeast nitrogen base without amino acids, 0.5% methanol, 40 µg/ml L-histidine, 40 µg/ml biotin, 1.5% agar). After colonies appeared, replicas were made on nitrocellulose circles, which were subsequently placed on glucose adaptation plates (6.7 g/l yeast nitrogen base without amino acids, 2.0% dextrose, 40 mg/l L-histidine, 40 µg/ml biotin, 1.5% agar) and incubated at 30°C for 12 to 14 hours, during which time the peroxisomes are degraded in normal cells. Glucose-adapted colonies on nitrocellulose circles were then assayed for alcohol oxidase (AOX) activity. Putative glucose-induced selective autophagy (gsa) mutant colonies were those retaining measurable AOX activity despite glucose adaptation. These positive colonies were picked from master plates for complementation, backcrossing and further characterization.

GS115 served as the parental strain for new strains in which the *P. pastoris* genes homologous to the *S. cerevisiae* vacuolar proteinases A and/or B (PrA and PrB) were disrupted by homologous recombination (M. A. G. Gleeson, personal communication). The putative changes in these vacuolar proteinase mutants are designated according to the *S. cerevisiae* nomenclature (Jones, 1991a) and named as follows: SMD1163 (his4, pep4, prb1; lacking PrA and PrB); SMD1165 (his4, prb1; lacking PrB); and SMD1168 (his4, pep4, lacking PrA). These strains were provided by L. V. Bennington of the Philips Petroleum Licensing Office. SMD1163 and SMD1168 were devoid of PrA and carboxypeptidase Y activities (data not shown).

**Antibody preparation and characterization**

Antibodies to *P. pastoris* AOX were prepared and characterized as previously described (Tuttle et al., 1993). Purified formate dehydrogenase (FDH) from *C. boidinii* (100 µg; Sigma Chemical Co.) was emulsified in Freund’s complete adjuvant and injected intradermally into a rabbit. A booster injection was administered, containing 50 µg FDH emulsified in Freund’s incomplete adjuvant, also injected intradermally. The immunological properties of FDH from *C. boidinii* and *P. pastoris* have been shown to be quite similar while the subunit size is somewhat larger in *P. pastoris* (Hou et al., 1982). Immunological analysis was performed on cell-free extracts, which were electroblotted on 7.5% SDS-polyacrylamide gels and then transferred to nitrocellulose. Immunoblotting was performed according to the enhanced chemiluminescence (ECL) method of Amersham (Arlington Heights, IL). On immunoblots, a single band of M, 45±10kDa was recognized in methanol-induced cellular extracts of *P. pastoris* (see Fig. 1). This band is in close agreement with that reported for FDH subunits of *P. pastoris* (Hou et al., 1982). Preabsorption of the antiserum with purified FDH greatly diminished the signal on immunoblots. Preabsorption with the same amount of an unrelated protein (AOX) had little effect on immunostaining (data not shown).

**Measurement of overall protein degradation**

The histidine auxotrophic yeast strains were precultured in YPD then inoculated at 1:30 into medium containing 6.7 g/l yeast nitrogen base without amino acids, 2% dextrose, 40 mg/l L-histidine, 40 µg/ml biotin, and 1 µC/ml [14C]valine (Amersham). Cultures were incubated for ~18 hours, then washed twice in 6.7 g/l yeast nitrogen base without...
amino acids. Individual cultures were then divided into two equal portions and incubated with 10 mM cold valine chase in the absence or presence of L-histidine (40 mg/ml) and in yeast nitrogen base without amino acids, 2% glucose and 4 µg/l biotin. Samples were collected on ice at 0, 5 and 8 hours of chase with the immediate addition of ice-cold trichloroacetic acid to a final concentration of 20% (w/v). The samples were incubated on ice for at least 1 hour, then centrifuged. The supernatant was aspirated and kept separate from the pellet, which was solubilized in 0.5 ml Scintigent (Fisher Scientific Co.). The radioactivity in the supernatant (acid-soluble counts) and solubilized pellet (acid-insoluble counts) was counted on a Beckman LS5000TD scintillation counter. Percentage degradation was calculated as the ratio of TCA-soluble counts at each time point to acid-insoluble (protein-associated) counts at 0 hour of chase, multiplied by 100 and normalized to 1 hour of chase.

Electron microscopy
Ultrastructural analysis was performed using a potassium permanganate fixation protocol (Veenhuis et al., 1983). Although this procedure fails to reveal cytoplasmic ground substances such as ribosomes, it effectively delineates membrane profiles and organelles in methylotrophic yeasts. Briefly, cells were harvested by centrifugation, washed in water, and fixed in 1.5% K MnO 4 in veronal-acetate buffer (0.3 mM sodium acetate; 0.3 mM sodium barbital, pH 7.6) for 20 minutes at room temperature. The specimens were dehydrated in increasing concentrations of ethanol, followed by 100% propylene oxide, then embedded in POLY/BED 812 (Polysciences, Inc., Warrington, PA). The blocks were sectioned and examined on a JEOL 100CX II transmission electron microscope.

Enzyme assays
Preparation of cell-free extracts during the various experiments was accomplished using glass beads as reported (Tuttle et al., 1993). Measurement of alcohol oxidase activity was performed with methanol as a substrate, producing hydrogen peroxide, in turn metabolized by horseradish peroxidase to oxidize 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), the generation of which was followed at 410 nm according to the procedures of Kato (1990). PrA assays, used to verify the absence of this activity in extracts from putative PrA null mutant strains, were performed according to Jones (1991b) and direct colony assays for carboxypeptidase Y (CPY) activity were according to Jones (1977).

RESULTS
Degradation of methanol-induced peroxisomes and cytosolic enzymes during glucose and ethanol adaptation
An essentially wild-type P. pastoris strain (GS115) was grown to stationary phase in medium containing 0.5% methanol and then allowed to adapt to media containing ethanol (0.5%) or glucose (2%). The kinetics of disappearance of peroxisomal and cytosolic methanol-metabolizing enzymes was measured during the first 6 hours of adaptation (Fig. 1). Ethanol adaptation resulted in a rapid decrease in (peroxisomal) AOX activity with a half-life of approximately 1 hour, while (cytosolic) FDH activity remained unchanged (Fig. 1, left panel). Glucose also induced the rapid loss of AOX activity but, unlike ethanol adaptation, the onset of degradation was not immediate (Fig. 1, right panel). After a lag period of approximately 1 hour, the rate of loss of AOX activity was equivalent to that observed during ethanol adaptation. Glucose also caused a concurrent decrease in FDH activity (Fig. 1, right panel). The decrease in AOX and FDH activities was accompanied by comparable reductions in AOX and FDH protein as determined by western blotting (Fig. 1, inset). We have previously shown that the turnover of the peroxisomal enzymes AOX and dihydroxyacetone synthase is minimal during stationary growth in methanol (Tuttle et al., 1993). Thus, losses of AOX and FDH are probably due to enhanced degradation, not suppressed synthesis.

We next examined the ultrastructural changes that occur after 1 hour of ethanol and glucose adaptation (Fig. 2). During ethanol adaptation, individual peroxisomes within a cluster were surrounded by one or more additional membranes of unknown origin (Fig. 2A, inset). This unique organelle was observed only during ethanol adaptation and was morphologically similar to an autophagosome described in nutrient-
stressed mammalian cells (for review see Dunn, 1994). Peroxisome-containing autophagosomes were observed in close proximity to or fusing with the yeast vacuole (Fig. 2B). Peroxisomes not sequestered within an autophagosome were not seen fusing with the vacuole. In addition, autophagosomes containing other cellular components such as mitochondria were not observed. Finally, peroxisomes were distinguishable within the vacuolar lumen, sometimes bound by additional membranes, presumed to be residual membranes of the autophagosome (Fig. 2C). During glucose adaptation, exten-

Fig. 2. Morphological characterization of sequestration of peroxisomes during ethanol and glucose adaptation. P. pastoris cells (GS115) were induced in methanol and then repressed for 1 hour in either ethanol (A-C) or glucose (D-F) at which time the cells were prepared for ultrastructural examination as described in Materials and Methods. (A) During ethanol adaptation, a profile of an individual peroxisome sequestered within an autophagosome (arrows) like the one shown here can be observed. Higher magnification of the autophagosome membrane within the box clearly reveals multiple membrane layers (inset). (B) A peroxisome-containing autophagosome (arrows) is shown fusing with the yeast vacuole (V). Those peroxisomes (P) without sequestering membranes (i.e. not sequestered within an autophagosome) were not observed fusing with the vacuole. (C) Intact peroxisomes (∗) still bound by additional membranes were also found within the vacuole, the site of degradation. (D) During glucose adaptation, peroxisomes (P) without additional membrane layers were routinely seen in close association with the yeast vacuole (V). (E) Extensions or arms of the vacuole were observed surrounding clusters of peroxisomes. (F) Peroxisomes (∗) in various stages of degradation could be distinguished within the vacuole. M, mitochondrion; Nu, nucleus. Bar, 0.5 µm; inset bar, 25 nm.
sions or arms of the vacuole were seen in close association with clusters of peroxisomes (Fig. 2D). These vacuolar extensions were observed partially or completely engulfing clusters of peroxisomes (Fig. 2E). Autophagosomes were not evident in these cells and the engulfment of mitochondria was not apparent. Profiles of peroxisomes in various stages of degradation could be seen within the vacuole (Fig. 2F).

**Protein synthesis is required for peroxisome degradation during glucose adaptation but not during ethanol adaptation**

Unlike the case of ethanol adaptation, peroxisome degradation was not evident during the first hour of glucose adaptation (see Fig. 1). It appears that those cellular events that initiate glucose adaptation require more time than those responsible for ethanol adaptation. This slow response suggested to us that protein synthesis may be required for glucose adaptation. In *P. pastoris*, protein synthesis as measured by the incorporation of $[^{35}\text{S}]	ext{methionine/cysteine}$ into cellular proteins was inhibited by 40% at cycloheximide concentrations of 0.1 mg/ml and was ~90% inhibited at 1.0 mg/ml. Cycloheximide at a concentration that inhibits 90% of protein synthesis did not affect ethanol-induced peroxisome degradation (Fig. 3A). However, the same concentration of cycloheximide abolished the degradation of AOX and FDH induced by glucose adaptation (Fig. 3A). Peroxisomes were in close proximity to but not within the vacuole even after 6 hours in glucose medium supplemented with cycloheximide (Fig. 3B). In addition, the vacuolar infoldings or arm-like extensions commonly observed during glucose adaptation (see Fig. 2D,E) were absent in the presence of cycloheximide. The fact that cycloheximide had dissimilar effects on peroxisome degradation under different nutrient stimuli suggests that in this case the drug is indeed acting by way of stopping protein synthesis, not pharmacologically. The data suggest that protein synthesis is required for the degradation of cytosolic FDH and peroxisomes during glucose adaptation but not during ethanol adaptation.

**Mutants defective in glucose-induced AOX degradation are able to degrade AOX during ethanol adaptation**

We have isolated mutant strains of *P. pastoris* that were defective in glucose-induced selective autophagy (gsa) of peroxisomes. To date, we have identified two complementation groups of gsa mutants (gsa1 and gsa2). Compared to parental GS115 cells (see Fig. 1), degradation of both AOX and FDH in response to glucose was inhibited by 70-90% in both mutant strains, while ethanol-induced AOX degradation proceeded normally (Fig. 4A). Since ethanol-induced degradation of AOX was unaffected in these mutants, and CPY and PrA activities were normal (data not shown), we suggest that these mutations did not affect vacuolar function (see below), but inhibited an event upstream of degradation. In order to evaluate whether peroxisome sequestration or degradation was altered in these mutants, we determined whether the peroxisomes accumulated outside or inside the vacuoles of glucose-adapted gsa2 cells. This was done by incubating gsa2 cells in methanol, then adapting them to glucose for 3 hours. The cells were fixed, embedded for sectioning, and their ultrastructure was examined (Fig. 4B). Peroxisomes were found almost exclusively outside the vacuole, suggesting that peroxisome entry into the vacuole was defective in these mutants. The fact that these gene products, GSA1 and GSA2, function in glucose adaptation but not ethanol adaptation provides further evidence of divergence between these two degradative pathways.

Fig. 3. The effects of cycloheximide on the degradation of methanol-induced enzymes and peroxisomes during ethanol and glucose adaptation. Methanol-induced GS115 cells were adapted to ethanol or glucose substrates in the absence or presence of cycloheximide (1 mg/ml). (A) Alcohol oxidase and formate dehydrogenase activities were determined in cell-free extracts obtained at 0 hour and 6 hours of adaptation. The values represent activities (mean + s.e.m.) measured at 6 hours and reported relative to that measured at 0 hour. (B) GS115 cells that had been adapted to glucose for 6 hours in the presence of cycloheximide were fixed and embedded for ultrastructural examination as described in Materials and Methods. Unlike untreated cells (see Fig. 2D), peroxisomes (P) were found outside the vacuole (V) of cycloheximide-treated cells. M, mitochondrion. Bar, 0.5 µm.
Proteinase mutants are defective in vacuolar degradation

Proteinases A and B (PrA, PrB) are required for the degradative function of the vacuole in *S. cerevisiae* (Takeshige et al., 1992; Teichert et al., 1989; Zubenko and Jones, 1981). The importance of PrA and PrB in vacuolar degradation was verified in *P. pastoris* by utilizing mutant strains in which the genes homologous to these proteinases have been disrupted (M. A. G. Gleeson, personal communication). PrA null mutants were deficient in PrA activity and exhibited negligible carboxypeptidase Y activity (data not shown), consistent with PrA having a role in the maturation of other vacuolar proteases. First, we studied the effect of the absence of functional PrA and/or PrB on overall protein degradation during amino acid starvation of histidine-requiring strains of *P. pastoris*. Various strains with proteinase deficiencies, SMD1163 (his4, pep4, prb1), SMD1165 (his4, prb1) and SMD1168 (his4, pep4), and the parental strain GS115 (his4), were metabolically labeled with [14C]valine during growth to stationary phase in glucose-enriched complete media. The cultures were then washed and chased with cold valine with and without histidine. Protein degradation, as quantified by the production of TCA-soluble radioactivity, was induced by histidine starvation in GS115. This nutrient stress-induced proteolysis was abolished in all presumptive vacuolar mutant strains (Fig. 5).

We next examined the ultrastructure of normal and mutant yeasts starved for a nitrogen source. The vacuoles of normal cells that were cultured in nitrogen-rich medium (data not shown) or nitrogen-depleted medium (Fig. 6A) were largely empty with infrequent vesicular bodies. Likewise, few vesicular bodies were present in proteinase mutant cells grown in the presence of nitrogen (Fig. 6B). However, numerous

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**Fig. 4.** Peroxisome sequestration and vacuolar degradation does not occur in glucose-adapted *gsa2* mutants. Methanol-induced *gsa2* cells were adapted to ethanol or glucose substrates. (A) Alcohol oxidase and formate dehydrogenase activities were determined in cell-free extracts obtained at 0 hour and 6 hours of adaptation. The values represent activities measured at 6 hours and reported relative to that measured at 0 hours. (B) *gsa2* cells that had been adapted to glucose for 3 hours were fixed and embedded for ultrastructural examination as described in Materials and Methods. Intact peroxisomes (P) were found adjacent to the vacuole (V). Profiles of the yeast vacuole (V) surrounding peroxisome clusters were also observed. M, mitochondrion. Bar, 0.5 µm.

**Fig. 5.** Protein degradation induced by histidine starvation is blocked in the absence of functional proteinases A and B. Parental *P. pastoris* (GS115: his4) cells were metabolically labeled with [14C]valine and then chased with medium containing cold valine in the absence of presence of histidine (HIS). Proteinase mutant strains, SMD1163 (his4, pep4, prb1), SMD1165 (his4, prb1) and SMD1168 (his4, pep4), were labeled with [14C]valine and chased in the absence of histidine. The release of acid-soluble radioactivity was measured, and the percentage of cellular protein degraded was calculated as a percentage of protein-associated (acid-insoluble) radioactivity at zero hour.
Pathways of autophagy in yeast

profiles of nondescript vesicular bodies and ribosomes accumulated in the vacuoles of nitrogen-starved SMD 1163 (Fig. 6C). Intact mitochondria were also observed within some vacuoles (Fig. 6C, inset). These observations suggest that cytoplasmic organelles can be sequestered within the vacuole of these mutants, but that the vacuole lacks the proteolytic capacity necessary to degrade these components. Furthermore, these data demonstrate that the PrA and PrB homologs are essential for the degradative function of the vacuole of *P. pastoris* and that the vacuole serves as an important site of starvation-induced degradation. Nitrogen starvation of the protease mutants results in accumulation of cytosolic contents within the vacuole. Vacuolar contents observed in protease mutants of *S. cerevisiae* have been referred to as autophagic bodies and are presumed to be sequestered by a process similar to autophagy (Takeshige et al., 1992; Baba et al., 1994). The

![Fig. 6](https://example.com/fig6.jpg)

**Fig. 6.** Effects of nitrogen starvation on parental and protease mutant *P. pastoris*. Parental GS115 (*his4*) (A) and mutant SMD1163 (*his4, pep4, prb1*) (B-D) cells were grown to stationary phase in YPD medium and washed in yeast nitrogen base medium without amino acids. The cells were then resuspended in media containing yeast nitrogen base without amino acids supplemented with 40 mg/l histidine, 4 µg/l biotin, and 2% glucose (B) or yeast nitrogen base without amino acids and ammonium sulfate, supplemented with histidine, biotin and glucose (A,C,D). The cells were incubated for 3 hours in these media, harvested, fixed, and processed for electron microscopy (see Materials and Methods). (A) The vacuoles of GS115 cells starved of nitrogen contained few inclusions. The vacuolar membrane contained many infoldings and a large finger-like protrusion (arrowheads). (C) Many nondescript vesicular bodies and an occasional mitochondrion (inset) were observed within the vacuole of the protease mutant that was deprived of nitrogen. These vacuoles contained many infoldings (open arrows) and profiles of ongoing microautophagy in which forming ‘autophagic bodies’ are still connected with the vacuolar membrane (filled arrow). (D) Upon cycloheximide treatment, the vacuoles of the nitrogen-starved mutants contained few vesicular bodies and few membrane infoldings. V, vacuole; M, mitochondrion; Nu, nucleus; er, endoplasmic reticulum. Bar, 0.5 µm; inset bar, 0.1 µm.
surface of the vacuole in *P. pastoris* cells maintained in nitrogen-deficient medium contained many protrusions and infoldings (Fig. 6A,C), suggesting ongoing microautophagy (Fig. 5A,C). This implies that these cytosolic components that accumulate within the vacuole of nitrogen-starved proteinase mutants may enter the vacuole by sequestration within invaginations of the vacuolar membrane as seen in microautophagy during glucose adaptation. We next examined whether the uptake of these components required protein synthesis. In the presence of cycloheximide, the accumulation of vesicular structures and amorphous material in the vacuoles of nitrogen-starved proteinase mutant cells was dramatically reduced when compared to untreated nitrogen-starved mutants (Fig. 6C,D). Cycloheximide also prevents the accumulation of autophagic bodies in *S. cerevisiae* (Takeshige et al., 1992). Infoldings (open arrow, Fig. 6C) and presumptive microautophagic events (filled arrow, Fig. 6C) were absent from those vacuoles in cycloheximide-treated cells (Fig. 6D). We suggest that the infoldings seen during glucose adaptation and nitrogen starvation in the absence of cycloheximide are not due to fixation artifacts, since they are not seen during ethanol adaptation or in the presence of cycloheximide. If indeed, these infoldings are indicative of ongoing microautophagy during nitrogen starvation, it would appear that cycloheximide effectively prevents this process in these cells.

**Proteinases A and B are required for the degradation of peroxisomes and FDH**

We next examined whether homologs of PrA and PrB were necessary for the degradation of peroxisomes and cytosolic FDH in *P. pastoris*. Protease mutant cells in which the genes homologous to PrA and PrB had been disrupted were grown to stationary phase in methanol and then adapted to ethanol or glucose medium. Unlike the parental cells (see Fig. 1), no significant degradation of AOX or FDH occurred in the mutant cells during either ethanol or glucose adaptation (Fig. 7A). This absence of proteolysis persisted for up to 6 hours of adaptation. Mutant cells that have undergone ethanol or glucose adaptation for 6 hours were found to accumulate intact peroxisomes within their vacuoles (Fig. 7B,C). However, unlike the effect of nitrogen starvation, mitochondria and other distinguishable organelles were not evident within the vacuoles of those proteinase mutant cells that were adapting to ethanol or glucose substrates. The data show that PrA and PrB are not required for the sequestration of peroxisomes, but are necessary for their vacuolar degradation during nutrient adaptation.

![Fig. 7.](image-url) Proteinase mutants are not able to degrade methanol-induced components during ethanol or glucose adaptation. Parental GS115 (*his4*) and mutant SMD1163 (*his4*, *pep4*, *prb1*) cells were grown in methanol, then adapted to ethanol or glucose. (A) Alcohol oxidase and formate dehydrogenase activity were determined at 0 hour and 6 hours of adaptation. The values represent the average + s.e.m. of at least three determinations done after 6 hours of adaptation and are reported as a percentage of that measured at 0 hour. After 6 hours of ethanol (B) and glucose (C) adaptation, the mutant cells were fixed and processed for ultrastructural examination (see Materials and Methods). (B,C) Intact peroxisomes (*) were observed within the vacuoles (V) of proteinase mutant cells. Arrowheads indicate the perimeter of the yeast vacuole. P, peroxisome; M, mitochondrion; Nu, nucleus. Bar, 0.5 µm.
We have shown biochemically, morphologically and genetically that there exist in *P. pastoris* at least two pathways for the entry of peroxisomes into the yeast vacuole, the site of degradation. These sequestration pathways differ in their nutrient signals, modes of sequestration of peroxisomes (i.e. micro- and macro-autophagy), requirements for protein synthesis, and abilities to recognize cytosolic proteins. These degradative pathways are similar in that peroxisomes are ultimately degraded within the yeast vacuole. In this report, we have identified mutant strains that are unable to sequester or degrade peroxisomes. Vacuolar entry of peroxisomes via microautophagy appears defective in *gsa1* and *gsa2* mutants, while yeasts lacking PrA and PrB are unable to degrade peroxisomes.

**Sequestration pathways**

In mammalian cells, cytoplasmic components are incorporated into lysosomes by micro- and macro-autophagy. In mammalian microautophagy, portions of cytosol are surrounded and engulfed within lysosomal invaginations in a non-selective manner (Mortimore et al., 1988). We have isolated mutant strains of *P. pastoris* that are defective in the degradation of peroxisomes and FDH during glucose adaptation (see Fig. 4). Ethanol adaptation occurs normally in this mutant, supporting our concept of divergent pathways of peroxisome degradation. Macroautophagy is acutely regulated by amino acid starvation and insulin and results in the sequestration of random portions of cytoplasm containing cytosol and organelles by portions of ribosome-free rough endoplasmic reticulum (Dunn, 1990a,b). The autophagic vacuoles formed in this way fuse with lysosomes, and acquire hydrolyases that rapidly degrade their contents. We report here that both micro- and macro-autophagy occur in *P. pastoris*. Glucose-induced peroxisomal uptake is effected directly by the vacuole as it extends finger-like protrusions, which surround clusters of several peroxisomes, and then incorporates them into its lumen. This process is similar to that seen during microautophagy. A distinctly different mode of vacuolar sequestration is revealed by morphological examination of *P. pastoris* undergoing ethanol adaptation. Ethanol adaptation induces a process whereby membrane layers of unknown origin closely surround individual peroxisomes within a cluster, resulting in a peroxisome sequestered in an autophagosome. Presumably, this autophagosome has the ability to recognize or be recognized by and fuse with the degradative vacuole. The contents of the autophagosome are deposited into the vacuolar lumen where they are ultimately degraded. This manner of delivery of cytoplasmic components to the lysosome is analogous to mammalian macroautophagy. Our data also suggest that protein synthesis is required for macroautophagy (glucose adaptation and nitrogen starvation), but not necessary for microautophagy (ethanol adaptation). The protein synthesis inhibitor cycloheximide prevented glucose-induced degradation of peroxisomes and FDH (see Fig. 3A). This observation, coupled with the inability of peroxisomes to enter the vacuole in the presence of cycloheximide (see Fig. 3B) leads us to conclude that the synthesis of certain protein(s) is required for the induction of the glucose-mediated degradative pathway. Cycloheximide has also been shown to inhibit the sequestration of ‘autophagic bodies’ in vacuoles of *S. cerevisiae* that have been nitrogen-starved (Takeshige et al., 1992). However, it remains uncertain whether these autophagic bodies arose from micro- or macro-autophagy or both.

The evidence suggests that both glucose-induced microautophagy and ethanol-induced macroautophagy of peroxisomes are selective. Although glucose induces the concomitant degradation of both peroxisomes and FDH, the degradation of mitochondrial F1ATPase β subunit is not enhanced during glucose adaptation (Tuttle et al., 1993). Ethanol-induced sequestration of peroxisomes appears to be selective as well, since FDH is not degraded and mitochondria are not seen within the vacuole of protease-deficient strains during ethanol adaptation. These results suggest that there exists a recognition event that initiates the selective sequestration of peroxisomes within autophagosome, and a lack of recognition of FDH. Selective degradation of peroxisomes has also been described in other methylotrophic yeasts, *H. polymorpha* and *C. boidinii*, during nutrient adaptation (Bormann and Sahm, 1978; Bruinenberg et al., 1982; Hill et al., 1985; Tuttle et al., 1993; Veenhuis et al., 1983). It is likely that recognition and/or sequestration and degradation of peroxisomes is mediated by the peroxisomal membrane. In fact, peroxisome assembly mutants of the methylotrophic yeast *H. polymorpha* have been isolated in which AOX crystalloids form in the cytosol without limiting membranes. These crystalloids allow growth on methanol but are not degraded during glucose adaptation (van der Klei et al., 1991). Recognition for sequestration of FDH during glucose adaptation is brought about by an unknown means that may involve either: (1) a mechanism for concentrating FDH in the region of peroxisomes so that they are engulfed along with peroxisomes (i.e. recognition by peroxisomes); or (2) recognition by the vacuole or a carrier protein and direct entry into the vacuole. We have shown that the degradation of FDH is suppressed when protein synthesis is inhibited by cycloheximide. Although we have yet to show that FDH remains in the cytosol of glucose-adapted cycloheximide-treated cells, it is likely that protein synthesis is required for vacuolar sequestration of FDH as was observed for peroxisomes (see Fig. 3). Indeed, several investigators have studied the selective degradation of soluble proteins in response to nutrient stress or adaptation. Catabolite inactivation of the soluble enzymes fructose-1,6-bisphosphate, phosphoenolpyruvate carboxykinase, isopropylmalate synthase, and cytoplasmic malate dehydrogenase (which are synthesized only in the absence of fermentable sugars) in response to glucose has been studied in *S. cerevisiae*. Holzer and Purwin (1986) have suggested that FBP is rapidly phosphorylated upon addition of glucose, targeting it for degradation. Chiang and Schekman (1991) have proposed that vacuolar recognition of cytosolic fructose-1,6-bisphosphatase requires the synthesis of a vacuolar import receptor similar to the lysosomal carrier postulated for mammalian cells (Dice and Chiang, 1989).

We have isolated mutant strains of *P. pastoris* comprising two complementation groups (*gsa1* and *gsa2*) that are defective in glucose-induced autophagy but support normal ethanol-induced autophagy. The ability of the vacuole to degrade peroxisomes is unimpaired, as indicated by the normal degradation rate of peroxisomes during ethanol adaptation (see Fig. 4A) and by the presence of normal levels of PrA and CPY activities in these strains (data not shown). This suggests that the proteins
that are mutated in these strains act upstream of the vacuole, assisting entry of the peroxisomes and FDH into the vacuole. Such proteins may be responsible for: (1) glucose signaling events; (2) peroxisome and FDH recognition; or (3) peroxisome and FDH sequestration. Although the identities and specific functions of GSA1 and GSA2 are unknown, these data support our hypothesis that the autophagic pathways initiated by glucose and ethanol follow different courses. P. pastoris is being used to elucidate the molecular basis of peroxisome biogenesis (Spong and Subramani, 1993). As we continue to isolate and characterize gsa mutant strains and identify those mutated proteins, we will be able to evaluate the functional roles of individual proteins in peroxisome degradation.

Vacular degradation

We examined the rôle of PrA and PrB in cellular proteolysis by comparing biochemical data and morphological observations of parental P. pastoris with strains carrying disrupted copies of the genes for these enzymes. The degradation of cellular proteins (see Fig. 5) and peroxisomes (see Fig. 7A) was dramatically inhibited in proteinase mutants, resulting in the accumulation of cellular components (see Fig. 6) and recognizable peroxisomes within the vacuole (see Fig. 7B). Thus, the data suggest that in P. pastoris these proteinases are required for protein degradation induced by nutrient starvation or adaptation. In S. cerevisiae, PrA and PrB serve as activators of other vacuolar hydrolases so that disruption of the genes for PrA or for PrA and PrB results in a drastic reduction in proteinase activities and overall protein degradation during nutrient stress (Teichert et al., 1989; Zubenko and Jones, 1981). Indeed, we have found that CPY activities are not detectable in mutants lacking PrA, consistent with an activation rôle for this enzyme in P. pastoris.

Glucose has been shown to stimulate the degradation of many cytosolic proteins in yeast. Such proteins may be degraded by either cytosolic or vacuolar mechanisms. We show here that the degradation of the cytosolic enzyme FDH induced by glucose was blocked in proteinase-deficient cells, suggesting that this enzyme is normally degraded in the vacuole (see Fig. 6). In S. cerevisiae, phosphoenolpyruvate carboxykinase and cytoplasmic malate dehydrogenase appear to be degraded independently of the vacuole and are presumably degraded by the cytosolic proteinase (Burlini et al., 1989; Funaguma et al., 1985). On the other hand, the degradation of fructose-1,6-bisphosphatase by S. cerevisiae is vacuolar, being dependent on functional PrA and PrB (Chiang and Schekman, 1991; Funaguma et al., 1985).

Our data indicate that PrA and PrB are essential for normal vacuolar degradation and that the vacuole is an important site of degradation during nutrient stress and catabolite inactivation in P. pastoris. In addition, the common point of convergence of the nutrient-induced different autophagic pathways is the vacuole. Furthermore, we have shown that while a proteolytically functional vacuole is required for the turnover of methanol-induced elements, entry into the vacuole is regulated by other cellular factors.

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REFERENCES


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