

Brefeldin A interferes with peroxisomal protein sorting in the yeast *Hansenula polymorpha*

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Abstract We have studied the effect of brefeldin A (BFA), a fungal toxin that interferes with coated vesicle formation, on the biogenesis of peroxisomes in the yeast *Hansenula polymorpha*. Addition of BFA (20 $\mu\text{g/ml}$) to cultures of *H. polymorpha* partially inhibited the development of peroxisomes and resulted in the reversible accumulation of newly synthesized peroxisomal membrane and matrix proteins at the endoplasmic reticulum. In contrast, BFA did not interfere with the selective degradation of peroxisomes. Taken together, our data suggest that the ER plays a crucial role in peroxisome biogenesis in *H. polymorpha*, possibly in the biosynthesis of the peroxisomal membrane.

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Key words: Brefeldin A; Peroxisome; Protein sorting; Yeast

1. Introduction

Peroxisomes are versatile subcellular organelles, which play a crucial role in various metabolic pathways [1]. In yeasts, peroxisomes are generally involved in the primary metabolism of the carbon and/or nitrogen source used for growth [2]. In the initial concept of peroxisome biogenesis, the organelles were thought to develop from the endoplasmic reticulum (ER). This view was predominantly based on morphological data which frequently showed a close association of peroxisomes with strands of the ER [3]. However, after the finding that peroxisomal proteins are synthesized in the cytosol on free polysomes, this concept changed into the current model which predicts that peroxisomes derive by fission from pre-existing ones [4]. Recently, several proteins (peroxins) essential for peroxisome biogenesis have been identified [5]. Unexpectedly, none of them showed significant homology to components of other protein translocation machineries (e.g. from mitochondria). In contrast two, Pex1p and Pex6p, are members of the protein family of AAA-ATPases and show homology to proteins involved in membrane fusion processes in eukaryotic cells (NSF/Sec18p) [6,7]. This may imply that vesicle-mediated processes may play a role in peroxisome biogenesis. In fact, several other morphological and biochemical findings provided indirect evidence for involvement of the ER in peroxisome biogenesis [8–11]. For this reason we tested whether brefeldin A (BFA) influences peroxisome biogenesis in the methylotrophic yeast *Hansenula polymorpha*. BFA is a fungal toxin which has been shown to prevent the formation of ER-derived coated vesicles [12,13]. We show that BFA indeed interferes with proper sorting of peroxisomal proteins

and resulted in the accumulation of these proteins at the ER. The details of these studies are presented in this paper.

2. Materials and methods

2.1. Organism and growth conditions

Hansenula polymorpha wild-type (WT) CBS4732, NCYC495, *leu1.1*, an *AOX* disruption mutant derived from this strain and NCYC495 *leu1.1* transformed with a plasmid encoding bacterial β -lactamase with or without a PTS1 [14] were grown at 37°C on mineral media supplemented with 0.5% glucose or 0.5% methanol in combination with either 0.25% ammonium sulfate or 0.25% methylamine [15]. In pulse-chase experiments [^{35}S]methionine (50 $\mu\text{Ci}\cdot\text{ml}^{-1}$; Amersham, UK) was added to cultures, 15 min after the addition of BFA (20 $\mu\text{g}\cdot\text{ml}^{-1}$), incubated for 5 min and chased with 2 mM cold methionine. Brefeldin A (BFA, Boehringer, Mannheim, Germany) was supplemented to *H. polymorpha* cultures at final concentrations ranging from 20 to 100 $\mu\text{g}\cdot\text{ml}^{-1}$ from a stock solution of 10 $\text{mg}\cdot\text{ml}^{-1}$ in methanol. For electron microscopy, BFA-treated cells were harvested and immediately resuspended into fixative at 0°C in order to prevent reversion of the BFA effects. For biochemical analyses sodium azide (0.02%) was added to the cultures 5 min before harvesting.

2.2. Biochemical methods and electron microscopy

Crude extract preparation [14], alcohol oxidase (AO) activity assays [16], determination of protein concentrations [14] and separation of AO monomers and octamers [17] were carried out as described before. Unlabeled AO protein was detected by Western blotting [18,19] using α -AO antibodies. ^{35}S -labeled AO protein was detected by fluorography after immunoprecipitation. The precipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel –80°C. Whole cells were fixed and prepared for electron microscopy and immunocytochemistry as described before [14].

3. Results

3.1. WT *H. polymorpha* is sensitive to BFA

In initial physiological experiments we observed that addition of BFA to batch cultures in the exponential growth phase on glucose resulted in strong retardation of growth (Fig. 1). A similar inhibitory effect was observed for cultures grown in methanol-containing media (data not shown). Electron microscopical analysis of such cells revealed that BFA caused drastic morphological alterations (Fig. 2). These were comparable to the effect of BFA on cells of a BFA-sensitive *Saccharomyces cerevisiae* strain [20] and in particular included the development of complex protrusions of the ER (Fig. 2). This effect was also observed in methanol-grown cells, although to a lesser extent as in glucose-grown cells (data not shown). The overall morphology of other cell organelles, including peroxisomes, remained virtually unaffected.

3.2. BFA results in mislocation of peroxisomal proteins to the ER

In an initial experiment aimed to analyze whether BFA affected normal peroxisome development in *H. polymorpha*,

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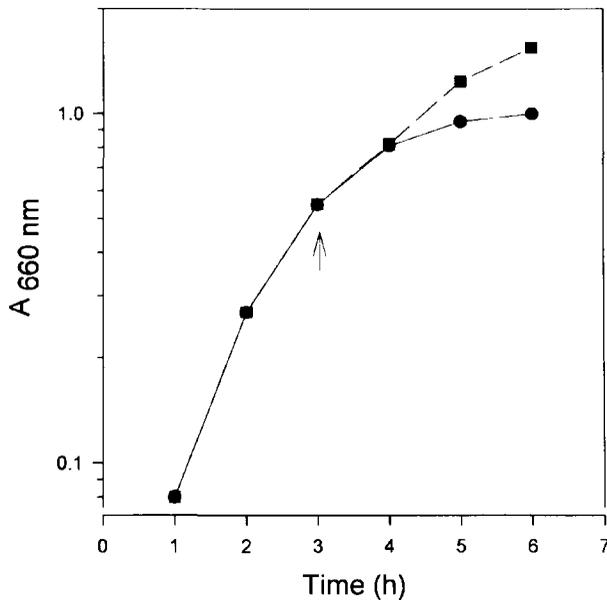


Fig. 1. Effect of BFA ($50 \mu\text{g}\cdot\text{ml}^{-1}$) on growth (measured as absorbance at 660 nm, A_{660}) of WT *H. polymorpha* cells in batch cultures on glucose. ■, control; ●, BFA-treated.

$20 \mu\text{g}\cdot\text{ml}^{-1}$ BFA was added to a batch culture of the organism in the mid-exponential growth phase on methanol. Immunocytochemical experiments revealed that alcohol oxidase (AO), a major peroxisomal matrix enzyme under these growth conditions, showed a dual location and was localized not only in peroxisomes, as expected, but also at the ER and the nuclear envelope (Fig. 3A,B). ER-labeling was not uniform; only distinct sections of the ER were labeled. In addition, AO labeling was occasionally found dispersed over the cytosol (Fig. 4A).

Two control experiments showed that the specificity of the α -AO antiserum used is extremely high and therefore, that the ER-located protein which was recognized by the α -AO antibody indeed represented AO protein. First, in BFA-treated glucose-grown cultures, in which AO synthesis is fully repressed, specific AO labeling was invariably fully absent, both on peroxisomes and at the ER. Similar results were

obtained in a second control experiment in which methanol-induced cells of an AO disruption strain were treated with BFA (data not shown). These data unequivocally demonstrate that the labeling patterns obtained are indeed significant and specific for AO protein.

Further immunocytochemical experiments revealed that not only AO, but also other peroxisomal matrix proteins (e.g. dihydroxyacetone synthase, catalase, amine oxidase and Pex8p [14]; Fig. 4C) accumulated at the ER in BFA-treated cells. In addition, ER-labeling was also observed when specific antibodies against the peroxisomal membrane proteins Pex3p [11] (Fig. 4B) and Pex14p [21] (Fig. 4E) were used in these experiments.

We subsequently studied the significance of the peroxisomal targeting signal (PTS) in the BFA-mediated sorting of matrix proteins to the ER. For this purpose we used *H. polymorpha* strains synthesizing bacterial β -lactamase fused to or lacking a PTS1 (-AKL-COOH) [14]. The β -lactamase protein lacking a PTS1 was exclusively found in the cytosol, both in BFA treated and in untreated control cells. However, in BFA-treated cells, synthesizing β -lactamase-AKL, the protein was found both in peroxisomes and in ER-like vesicular structures (Fig. 4D). In untreated controls the fusion protein was only found inside peroxisomes. These results suggest that the BFA-induced delivery of proteins to distinct regions of the ER is dependent on a functional peroxisomal targeting signal. This view was strengthened by experiments in which we used antibodies which cross-react with cytosolic and mitochondrial Hsp70 [22]. Both in BFA-treated and in untreated control cells labeling was exclusively found at the normal location in the cytosol and mitochondria (Fig. 4G); ER-labeling was never observed. As further control also the location of acid phosphatase was determined. In WT *H. polymorpha* acid phosphatase is located in the vacuole and – only partially – secreted. In untreated cells, α -acid phosphatase specific labeling was located on the vacuole. However, in BFA-treated cells labeling was also observed at the ER and the nuclear envelope (Fig. 4F). This finding is in line with earlier reports which showed that BFA treatment of eukaryotic cells results in the fusion of different compartments of the endomembrane system [12,23].

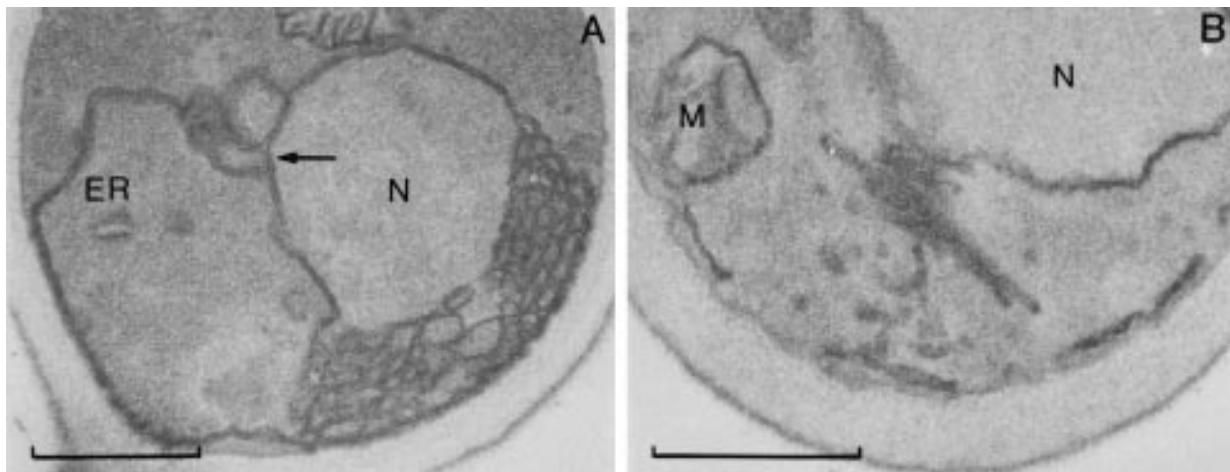


Fig. 2. Morphology of glucose-grown *H. polymorpha* WT cells, incubated in the presence of BFA ($50 \mu\text{g}\cdot\text{ml}^{-1}$) for 1 h. Typical examples are given of the excessive development of ER protrusions forming complex membranous structures (A,B KMnO_4), which are generally continuous with the nuclear envelope (A, arrow). Abbreviations: ER, endoplasmic reticulum; N, nucleus; M, mitochondrion; P, peroxisome. Bar = $0.5 \mu\text{m}$.

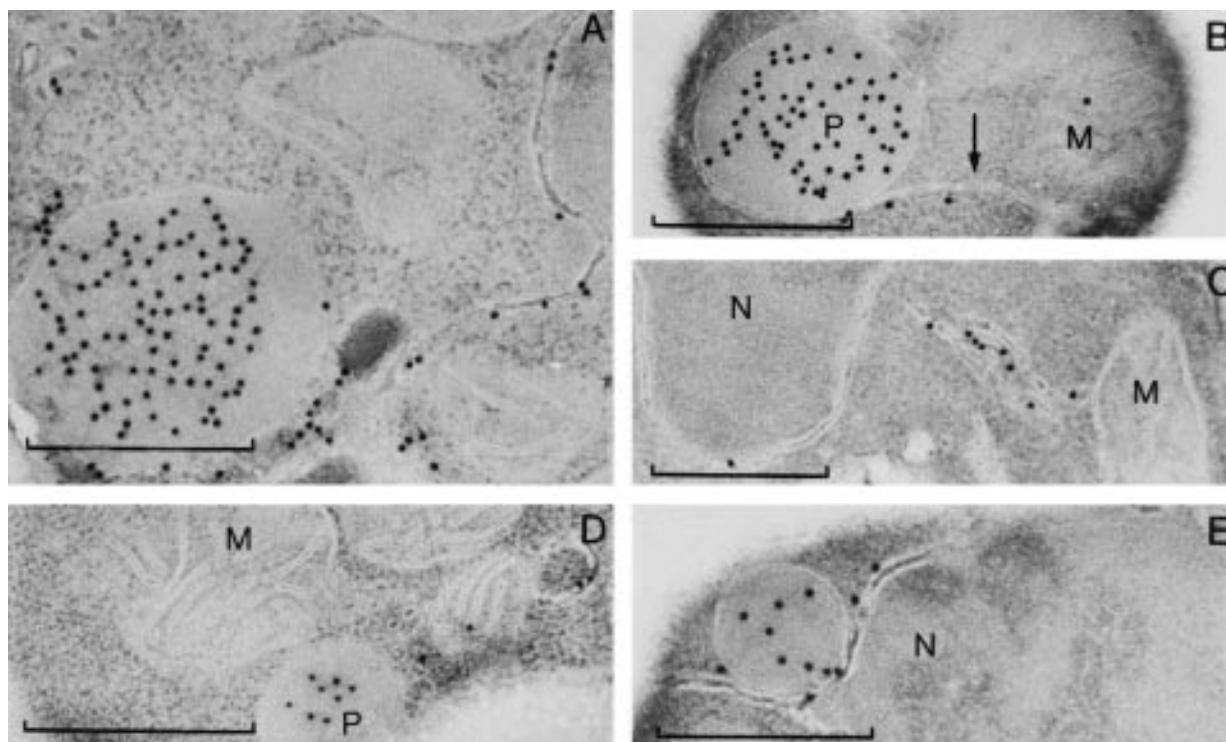


Fig. 3. Immunocytochemical demonstration of AO protein in methanol-grown *H. polymorpha* cells, incubated with BFA ($20 \mu\text{g}\cdot\text{ml}^{-1}$) for 1 h. Using α -AO antibodies, labeling is evident on both peroxisomes and ER-like membranes (A,B; arrow B). The labeling is generally seen on specific strains of the ER, which are closely associated with peroxisomes and/or continuous with the nuclear envelope (compare E). C: Labeling on ER-like membranes 1 h after the shift of cells from glucose to methanol in the presence of BFA; D,E: 3 h after the shift both induced peroxisomes and ER-like strands are labeled (A–E; glutaraldehyde, uranyl acetate).

Because BFA is known to induce fusion of different sub-cellular compartments, AO labeling at the ER could be due to fusion of peroxisomes with the ER. To study the origin of the peroxisomal proteins accumulating at the ER, we carried out the following experiment. First, cells were pre-grown on methanol/methylamine resulting in AO and amine oxidase (AMO)-containing peroxisomes. The cells were collected by centrifugation and incubated in mineral media lacking carbon and nitrogen sources (30 min, 37°C) to deplete the cells from AMO mRNAs. Subsequently, the cells were transferred to methanol/ammonium sulfate containing media supplemented with BFA ($20 \mu\text{g}\cdot\text{ml}^{-1}$). Under these conditions AO synthesis is induced but AMO synthesis is fully repressed (by ammonium) [24]. Immunocytochemistry revealed that after 1 h of incubation in the presence of BFA, AMO protein was exclusively located in the peroxisomal matrix, whereas AO protein was again found in peroxisomes and the ER. Thus, the BFA-mediated mislocation of AO protein does not result from

fusion of the ER with peroxisomes. Apparently, only newly synthesized peroxisomal proteins are sorted to the ER.

We subsequently tested whether the AO protein synthesized during BFA treatment is quantitatively mistargeted to the ER. For this purpose cells from exponentially growing cultures on glucose were transferred to methanol-containing media supplemented with BFA at concentrations ranging from 20 to $100 \mu\text{g}\cdot\text{ml}^{-1}$. In the glucose-grown inoculum cells only one or few small peroxisomes are present, which lack AO protein because of glucose repression. Upon transfer to methanol-medium AO synthesis is induced (see also Table 1). After 1 h of incubation in the presence of BFA newly synthesized AO protein was found at the ER (Fig. 3C). However, after 3 h AO protein was detected inside peroxisomes as well (Fig. 3D,E). Thus, BFA induces the mistargeting of only a portion of the newly synthesized peroxisomal matrix proteins.

The reversibility of BFA-induced mistargeting of peroxisomal proteins was evident after analysis of cells which were collected by centrifugation and resuspended in fresh medium without BFA. Already after 10 min of incubation in fresh media AO labeling at the ER was strongly reduced and invariably undetectable after 30 min of incubation in fresh media. In these cells AO protein was again only found inside peroxisomes (data not shown).

In order to determine the biochemical properties of AO synthesized in the presence of BFA, we analyzed AO protein in crude extracts prepared from cells pre-grown on glucose and shifted for 1 h to methanol-medium in the presence of BFA (20 – $100 \mu\text{g}\cdot\text{ml}^{-1}$). Enzyme activity measurements revealed that in BFA-treated cells the AO activities were lower compared to those found in extracts from identically grown

Table 1
Specific alcohol oxidase (AO) activities in relation to BFA concentrations in the growth medium

Concentration of BFA ($\mu\text{g}\cdot\text{ml}^{-1}$)	Specific AO activity ($\text{mU}\cdot\text{mg protein}^{-1}$)
0	44
20	27
50	14
100	9

Glucose-grown cells were transferred to methanol-containing media supplemented with different concentrations of BFA. After 1 h of incubation, the cells were harvested and used to determine the specific alcohol oxidase activities.

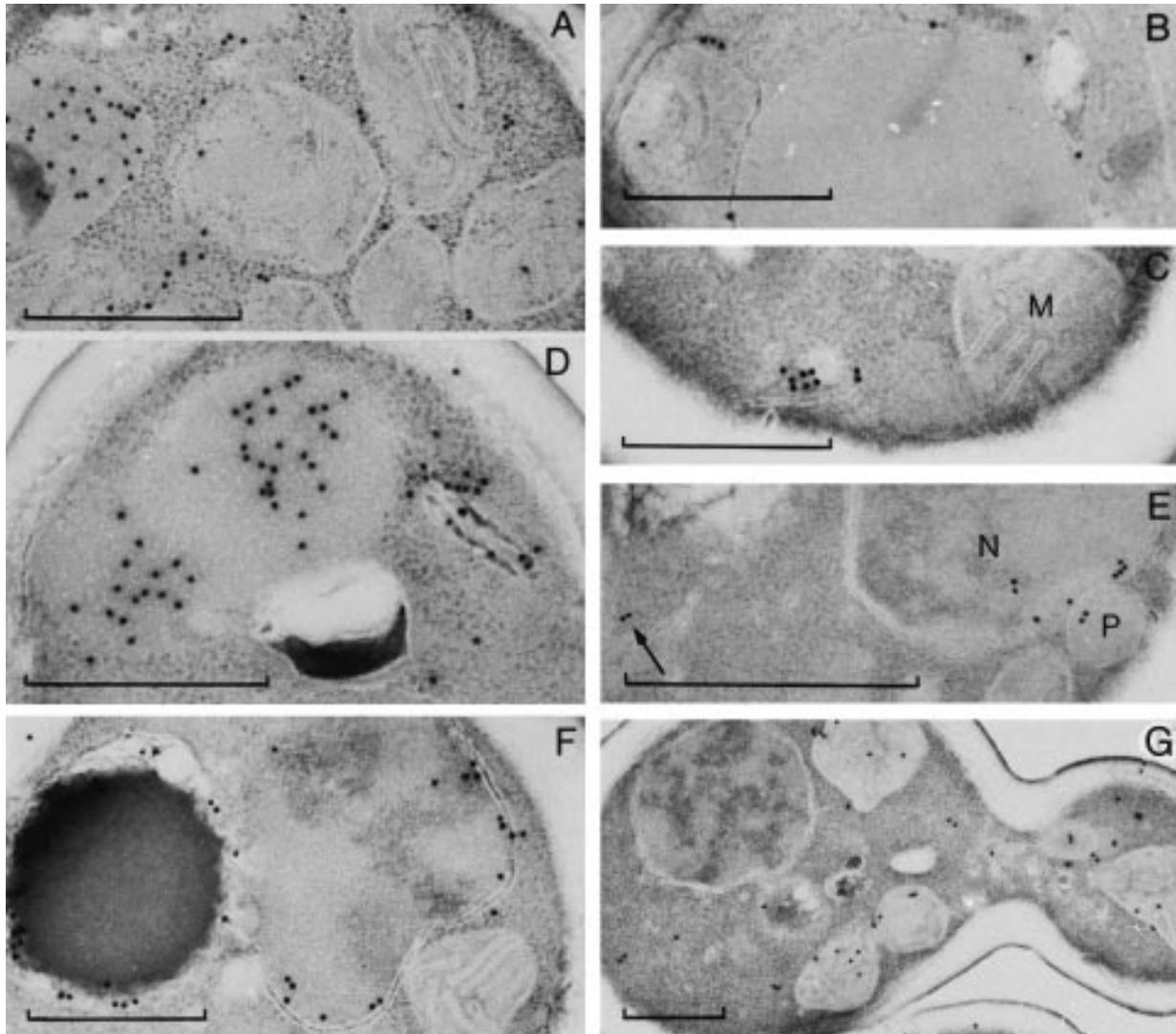


Fig. 4. Immunocytochemical localization studies in BFA-treated *H. polymorpha* cells using various antibodies against *H. polymorpha* proteins. Apart from peroxisome- and ER-bound labeling (shown in Fig. 3), α -AO specific labeling was occasionally also found in the cytosol (A). The integral peroxisomal membrane protein Pex3p (B), the membrane associated protein Pex8p (C) and Pex14p (E) were localized both on the peroxisomal membrane and at the ER. D: BFA-treated cells which synthesize bacterial β -lactamase fused to a PTS1. Using α - β -lactamase antibodies labeling is observed both on peroxisomes and ER. Using α -acid phosphatase antibodies labeling was found in the vacuole and the nuclear envelope (F). In controls, using α -hsp70 antibodies which recognizes both cytosolic and mitochondrial *H. polymorpha* hsp70s labeling was confined to mitochondria and the cytosol (G).

control cells (Table 1). We then studied whether BFA affects the assembly of octameric AO enzyme. As shown in Fig. 5A, both in BFA-treated cells and untreated control cells bulk of the AO protein synthesized was in the octameric conformation.

Pulse chase experiments revealed that also the kinetics of AO oligomerization were identical in BFA-treated and untreated controls (Fig. 5B). Thus, BFA does not significantly affect oligomerization of AO. The reduced AO enzyme activities in BFA-treated cells are most probably due to decreased AO expression levels, possibly caused by the retardation of growth (see also Fig. 1).

3.3. Glucose-induced peroxisome degradation is not prevented by BFA

In *H. polymorpha* a shift of cells from methanol to excess glucose results in the active degradation of peroxisomes by autophagocytosis [25]. The initial stage of this process is se-

questration of the organelles to be degraded from the cytosol before fusion with the vacuole where degradation occurs. To study whether BFA affects this process, $20 \mu\text{g}\cdot\text{ml}^{-1}$ BFA was added to a batch culture in the mid-exponential growth phase on methanol, incubated for 5 min and subsequently supplemented with excess glucose. Enzyme activity measurements and Western blot analysis indicated that AO activity and protein rapidly declined both in BFA-treated and in untreated control cultures. This result was confirmed in subsequent electron microscopical analyses, which revealed that glucose-induced peroxisome degradation was not affected by BFA (data not shown).

4. Discussion

In this study we showed that BFA affects protein sorting to peroxisomes resulting in the accumulation of these proteins at the ER. Morphological and biochemical effects caused by

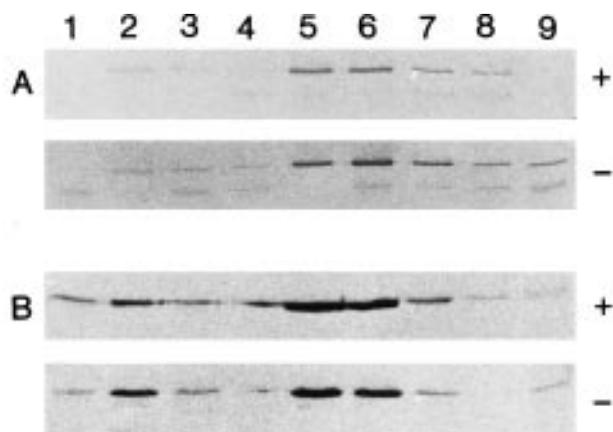


Fig. 5. Separation of octameric (fractions 5, 6) and monomeric AO (fraction 2) protein by sucrose density centrifugation of crude extracts prepared from cells treated with BFA ($20 \mu\text{g}\cdot\text{ml}^{-1}$; +) or from untreated controls (-). A: Cells were shifted from glucose- to methanol-containing media either in the presence (+) or absence (-) of BFA and incubated for 1 h. Crude extracts were subjected to sucrose-density centrifugation. Western blots, prepared from the different fractions decorated using α -AO antibodies, reveal that both in the absence and presence of BFA almost all AO protein found is octameric. Equal volumes of each fraction were loaded per lane. To determine the kinetics of AO oligomerization a pulse-chase experiment was performed (B). BFA was added to cells growing exponentially on methanol. After 15 min of incubation, a pulse of [^{35}S]methionine was given for 5 min. After 40 min of incubation samples were taken and used to prepare sucrose-gradients. Equal volumes of each fraction were subjected to immunoprecipitation using α -AO antibodies, followed by SDS-PAGE and fluorography. The results indicate that the kinetics of AO oligomerization were not significantly altered in BFA-treated cell.

BFA are, without exceptions, ascribed to the inhibition of binding of cytosolic coat proteins to membranes of the ER and Golgi. Binding of coat proteins to membranes requires a small GTP-binding protein, designated ADP-ribosylation factor (ARF; reviewed in [26]). Exchange of the ARF-bound GDP for GTP results in the binding of ARF and cytosolic coat proteins to membranes. BFA inhibits the GDP/GTP exchange activity for ARF and, as a consequence, prevents the formation of coated vesicles [12,13]. It is likely that BFA-induced accumulation of peroxisomal proteins at the ER also results from the prevention of the formation of vesicles and that a BFA-sensitive ARF-like protein is involved. It has been shown before that indeed different organelle-specific target proteins for BFA exist in eukaryotic cells [27]. In *Saccharomyces cerevisiae* three ARF genes have been identified; two of them are involved in protein secretion, whereas the function of the third one (ARF3) remained elusive so far [28]. In fact, ARF3 would be a possible candidate to encode an ARF involved in peroxisome biogenesis, because it shares properties with genes involved in peroxisome biogenesis (PEX genes): the expression of ARF3 is repressed by glucose and deletion of the gene is not lethal [28]. We have not been able to unequivocally determine, whether the BFA-induced mistargeted peroxisomal matrix proteins are actually located in the ER lumen or bound to its cytosolic surface. Protease protection assays, performed on subcellular fractions, were invariably poorly reproducible and the outcome difficult to interpret. Part of these problems might be explained by the fact that BFA only partially blocked peroxisomal matrix protein import and that the effect of BFA was highly reversible. This

might explain why biochemically no significant effects of BFA could be demonstrated (e.g. activation and oligomerization of AO were unaltered upon BFA-treatment). We also found no indications that mistargeted peroxisomal matrix proteins were modified (e.g. glycosylated), a process which is likely to occur when these proteins would enter the ER lumen. This therefore suggests that the matrix proteins most probably were bound to the cytosolic surface of the ER membrane.

Our present view on the principles of peroxisome biogenesis in *H. polymorpha* are included in the model, depicted in Fig. 6. This model predicts that vesicles are formed at specialized regions of the ER which fuse with peroxisomes resulting in growth of the organelle and incorporation of membrane components (proteins/lipids). We speculate that only a subset of the peroxisomal membrane proteins (PMP) are sorted to peroxisomes via the ER (PMB: peroxisomal membrane proteins involved in peroxisome biogenesis). When the formation of these vesicles is blocked by BFA, these PMBs will predominantly accumulate at the ER (e.g. Pex3p and Pex14p). Likely PMB candidates include *H. polymorpha* Pex3p and Pex14p, which were shown to accumulate at the ER when they are overproduced [11,21]. Moreover, *S. cerevisiae* Pas21p was delivered to the plasma membrane when the last 55 amino acids were deleted [10]. One possible explanation for this phenomenon is that Pas21p is initially targeted to the ER and subsequently delivered to peroxisomes by vesicular transport; in this process the last 55 amino acids may be essential for a correct routing to peroxisomes and/or to prevent that the protein enters the secretory pathway. We anticipate that not

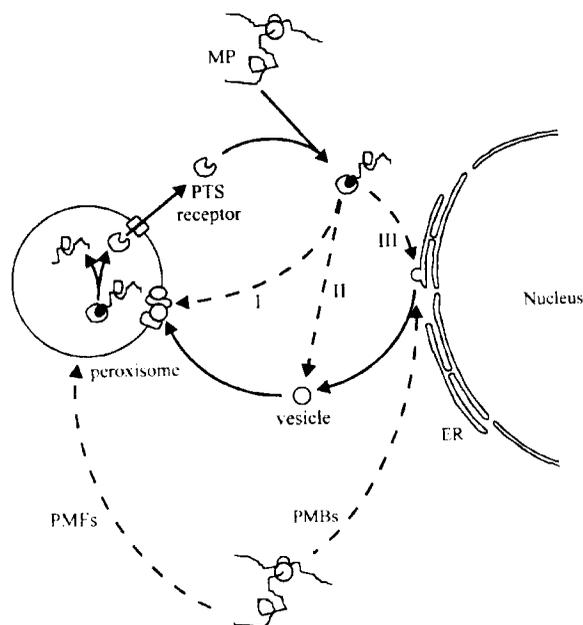


Fig. 6. Hypothetical model of peroxisome biogenesis in *H. polymorpha*. Peroxisomal proteins are synthesized on free polysomes in the cytosol. Membrane proteins involved in peroxisome biogenesis (PMBs, e.g. HpPex3p) are sorted to the ER and incorporated into vesicles which are transported to the growing peroxisome. Membrane proteins involved in peroxisome functioning (PMFs, e.g. transporters) may be sorted directly to the organelle. Matrix proteins (MP) are recognized by PTS-receptor proteins in the cytosol and subsequently either transported directly to a peroxisomal docking site (I), a putative transport vesicle (II) or the ER (III). After import into the peroxisomal matrix the receptor and its cargo molecule dissociate followed by recycling of the receptor.

all PMPs are sorted via the ER. Functional membrane proteins (e.g. proteins involved in transport of solutes, for instance *Candida boidinii* PMP47 [29]) may be sorted directly from the cytosol to the peroxisomal membrane (PMF: peroxisomal membrane protein involved in peroxisome functioning).

For sorting of peroxisomal matrix proteins (designated MP) our model envisages different possibilities. Matrix proteins may, upon synthesis in the cytosol and recognition by a PTS-receptor be targeted directly to the peroxisomal membrane (pathway I), to a transport vesicle (pathway II) or to the ER (pathway III). Several recent papers favour pathway I (reviewed in [30]). In this concept the PTS-receptor, bound to its cargo, interacts with the peroxisomal membrane at a docking site. For the subsequent step two alternative pathways have been proposed. One predicts that receptor/cargo complex enters the peroxisomal matrix, where dissociation occurs. The PTS-receptor is then exported to the cytosol, from where it can shuttle the next protein molecule into the organelle (see Fig. 6). The alternative is that the receptor already dissociates from the PTS protein at an earlier stage, namely at the peroxisomal surface. In this model the receptor shuttles between the cytosol and the docking site on the peroxisomal surface (see [30]). For *H. polymorpha* evidence has been obtained that the PTS1-receptor enters the organellar matrix [31]. Two putative docking proteins, Pex13p and Pex14p, have recently been identified [21,32–35]. These proteins have been shown to physically interact with the PTS-receptors. As a consequence they can indirectly bind PTS-cargo proteins. We propose that Pex13p and Pex14p are PMBs and thus accumulate at the ER upon BFA-treatment, because vesicle formation is prevented. Indeed, we found Pex14p at the ER membrane in BFA-treated *H. polymorpha* cells; information on HpPex13p is not available, because the gene has not been identified yet.

If pathway I (Fig. 6) is in fact the normal import pathway, why would matrix proteins become ER-associated in BFA-treated cells? One possibility is that the accumulation of Pex14p at the ER upon BFA-treatment may result in the formation of an artificial PTS-receptor docking site. This would readily explain why both peroxisomal membrane and matrix proteins accumulate at the ER under these conditions. Moreover, it would also explain why only a portion of the newly synthesized proteins are delivered to the ER, because next to BFA-induced docking sites at the ER, pre-existing peroxisomal docking sites are available as well.

An alternative explanation for the ER location of matrix proteins in BFA-treated cells (pathways III, Fig. 6) includes that these proteins are not transported directly, but are delivered to the target organelle together with membrane components. This view is in line with the notion that large amounts of matrix proteins can only be incorporated in the organelle when simultaneously the organellar volume is increased. In this concept peroxisomal matrix proteins are first targeted to the ER and accumulate with PMBs in or at transport vesicles, which migrate to developing peroxisomes. In these processes Pex1p and Pex6p – two AAA-ATPases essential for peroxisome biogenesis in yeasts – may play a role. According to this model inhibition of vesicle formation by BFA obviously results in accumulation of matrix proteins at/in the ER. Hypothetically, matrix proteins could of course also interact with or be incorporated in the transport vesicle after it has been formed at the ER (pathway II).

Our model implies that successful import of components into peroxisomes is only dependent on the availability of an exposed PTS and therefore also offers an explanation for the finding that oligomeric proteins and even gold particles coated with PTS-peptides are incorporated into peroxisomes [36–38]. The molecular mechanisms of import (e.g. fusion events or phagocytosis-like processes) are still completely unknown.

Which of the three pathways for matrix protein import proposed in Fig. 6 is correct is also speculative. However, all include a temporal association of matrix proteins via PTS-receptors with PMBs, which are sorted to peroxisomes via the ER. As a consequence a block in the formation of ER-derived vesicles will invariably result in accumulation of membrane and matrix proteins at the ER, independent of which pathway for matrix proteins is actually correct.

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