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Peroxisomes in the methylotrophic yeast *Hansenula polymorpha* do not necessarily derive from pre-existing organelles

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We have identified two temperature-sensitive peroxisome-deficient mutants of *Hansenula polymorpha* (ts6 and ts44) within a collection of ts mutants which are impaired for growth on methanol at 43°C but grow well at 35°C. In both strains peroxisomes were completely absent in cells grown at 43°C; the major peroxisomal matrix enzymes alcohol oxidase, dihydroxyacetone synthase and catalase were synthesized normally but assembled into the active enzyme protein in the cytosol. As in wild-type cells, these enzymes were present in peroxisomes under permissive growth conditions (≤ 37°C). However, at intermediate temperatures (38–42°C) they were partly peroxisome-bound and partly resided in the cytosol. Genetic analysis revealed that both mutant phenotypes were due to monogenic recessive mutations mapped in the same gene, designated *PER13*. After a shift of *per13-6* cells from restrictive to permissive temperature, new peroxisomes were formed within 1 h. Initially one—or infrequently a few—small organelles developed which subsequently increased in size and multiplied by fission during prolonged permissive growth. Neither mature peroxisomal matrix nor membrane proteins, which were present in the cytosol prior to the temperature shift, were incorporated into the newly formed organelles. Instead, these proteins remained unaffected (and active) in the cytosol concomitant with further peroxisome development. Thus in *H. polymorpha* alternative mechanisms of peroxisome biogenesis may be possible in addition to multiplication by fission upon induction of the organelles by certain growth substrates.

**Key words:** Hansenula polymorpha/peroxisome biogenesis/peroxisome-deficient mutant/peroxisomal membrane proteins/yeast

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**Introduction**

In yeasts, microbodies (peroxisomes, glyoxysomes) play a crucial role during growth of cells on a variety of carbon and nitrogen sources (Veenhuis and Harder, 1987). In order to gain more insight into the mechanisms involved in peroxisome biogenesis and function in the methylotrophic yeast *Hansenula polymorpha*, a collection of mutants affected in peroxisome assembly was generated from this organism (Cregg et al., 1990; Titorenko et al., 1993). Within this collection three different morphological phenotypes are identified, namely Per+ mutants, characterized by the complete absence of recognizable peroxisomal structures (13 complementation groups; Cregg et al., 1990; Veenhuis, 1992; Titorenko et al., 1993); Pim+ mutants, which contain small peroxisomes, while the bulk of the peroxisomal matrix proteins resides in the cytosol (five complementation groups; Waterham et al., 1992b) and Pss+ mutants, characterized by peroxisomes with aberrant crystalline matrix substructures (two complementation groups; Titorenko et al., 1993).

In both Per+ and Pim+ mutants peroxisomal matrix enzymes are synthesized at wild-type (WT) levels which are correctly assembled and activated in the cytosol (Sulter et al., 1990; van der Klei et al., 1991b). As a consequence, various peroxisomal functions (e.g. peroxisomal nitrogen metabolism) may effectivly occur in the cytosol of these mutants (Sulter et al., 1990). However, growth on methanol appeared to be strictly dependent on the presence of intact peroxisomes (van der Klei et al., 1991a).

Here we report the isolation and characterization of temperature-sensitive (ts) peroxisome-deficient mutants of *H. polymorpha*, which completely lack peroxisomes at restrictive temperatures, but show the WT phenotype (and contain normal peroxisomes) at permissive growth conditions. It will be shown that these mutants provide attractive model systems for studies on peroxisome biogenesis since they allow precise adjustment of the rate of peroxisome development from zero to WT levels by adaptations in the growth temperature. The reintroduction of peroxisomes, occurring after a shift of cells from restrictive to permissive growth conditions, has been investigated in detail. In particular the fate of mature cytosolic peroxisomal matrix and membrane proteins, already present prior to the temperature shift of cells, has been studied.

**Results**

**Mutant isolation and genetic analysis**

Screening of ~5000 yeast colonies yielded 2923 methanol-non-utilizing (Mut-) mutants at frequencies ranging from 58 to 64% in different rounds of NTG mutagenesis and subsequent nystatin enrichments. From these Mut- mutants 97 strains (± 3%) were identified as ts, in that they showed normal growth on methanol plates at 30°C (Mut+ phenotype), whereas growth on this compound was impaired at 43°C (Mut+ phenotype). Subsequent screening of the ts Mut- mutants by phase contrast light microscopy and electron microscopy, revealed seven mutant strains affected in different aspects of peroxisome biogenesis/assembly. Two of these mutants, namely strains ts6 and ts44, showed a Per+ phenotype at 43°C (and lacked peroxisomes) but displayed WT properties (and contained normal peroxisomes) at 30°C (Figure 1A and B). To remove possible hidden...
mutations both mutant strains were backcrossed three times with mutual isogenic auxotrophic H. polymorpha NCYC 495 strains and subsequently subjected to extensive genetic analysis.

Random spore analysis of heterozygous diploids containing WT and mutant alleles showed that both the temperature sensitivity and the Per− phenotype in mutants ts6 and ts44 are caused by monogenic, recessive mutations. Complementation and linkage analysis indicated that both ts mutants contained allelic mutations and therefore belong to the same complementation group. Since both mutants complemented representative Per− and Pim− mutants from all complementation groups previously identified (Waterham et al., 1992b; Titorenko et al., 1993), they belong to a new complementation group designated PER13. Mutant strain per13-6ts was subjected to a detailed biochemical and morphological analysis, which is described below.

**Growth, enzyme activities and enzyme location**

Growth experiments carried out in liquid cultures indicated that at permissive temperatures (<37°C) mutant per13-6ts showed WT properties (Mut+ phenotype), while at restrictive temperatures (43°C) growth on methanol was impaired (Mut− phenotype). However, growth on other

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**Fig. 1.** Cells of per13-6ts grown at various temperatures in glucose–methanol-containing media. Cells grown at permissive temperatures (30°C) contain many peroxisomes (A). These organelles are absent in cells grown at 43°C which contain a large cytosolic AO crystalloid (B; protoplast; glutaraldehyde–OsO4). The insets in panels A and B represent phase contrast light micrographs of such cells. (C and D) Dual presence of peroxisomes and cytosolic aggregates (*) in cells grown at intermediate temperatures, namely 39°C (C) and 41°C (D). The size of the peroxisomes (indicated by an arrow in D) decreases with increasing growth temperatures. The crystalline nature of the AO crystalloids shown in panels C and D is not well resolved due to the KMnO4 fixation. Inset panel C: detail of glutaraldehyde–OsO4 fixed protoplasts to show the crystalline structure of the intact peroxisomes in cells grown at 39°C. P, peroxisome; V, vacuole; N, nucleus. Bars represent 0.5 μm.
substrates including various organic nitrogen sources, the metabolism of which is mediated by peroxisome-borne enzymes in WT strains, was not affected (Table I). Therefore, at restrictive temperatures per13-6\(^b\) behaved in a fashion identical to constitutive peroxisome-deficient (per) mutants of *H. polymorpha* which have been described previously (Cregg et al., 1990; Sulter et al., 1990; van der Klei et al., 1991b). The Per\(^-\) phenotype of mutant per13-6\(^b\) was confirmed in additional biochemical and ultrastructural studies which showed that under restrictive growth conditions various peroxisomal matrix enzymes were normally synthesized but located in the cytosol (results not shown; for detailed information on Per\(^-\) phenotype, see Sulter et al., 1990; van der Klei et al., 1991b; Veenhuis, 1992). As expected, fully deregressed cells of per13-6\(^b\), grown in continuous culture on glucose—methanol mixtures at 43°C, lacked peroxisomes. Instead they contained a large cytosolic crystallloid composed of alcohol oxidase (AO) protein (Figure 1B), in which the bulk of the other major matrix constituent dihydroxyacetone synthase (DHAS) was incorporated, while catalase (CAT) was present in soluble form in the cytosol (not shown; for details see van der Klei et al., 1991b). Fully deregressed cells of per13-6\(^b\), grown at permissive temperatures (<37°C), showed the WT phenotype in that they contained many cuboid peroxisomes (Figure 1A). Unexpectedly, in steady state cultures grown at intermediate temperatures (between 38 and 42°C), intact peroxisomes were present together with cytosolic AO crystalloids. (Immuno)cytochemical experiments indicated that under these conditions the different matrix proteins tested, namely AO, DHAS and CAT, were located both in peroxisomes and in the cytosol and therefore these cells displayed a Pim\(^-\) phenotype (Waterham et al., 1992b). The volume fraction of peroxisomes varied with the temperature and increased with decreasing temperatures (Figure 1C and D). As indicated, peroxisomes were completely absent at 43°C, whereas cells grown at 35°C showed a WT phenotype; at 40°C the peroxisomal volume fraction amounted to ~50% of that of cells grown at the permissive temperature (35°C). Interestingly, the average size of individual peroxisomes in cells from steady state cultures grown at various temperatures also varied and increased with decreasing temperatures. Large cuboid peroxisomes were predominantly observed in cells grown at permissive temperatures (Figure 1A); at intermediate temperatures the organelles were predominantly rounded or of irregular shape; typical examples are shown in Figure 1C and D.

In conclusion, our results demonstrate that at restrictive temperatures (43°C) the phenotype of mutant per13-6\(^b\) is identical to constitutive Per\(^-\) mutants, at intermediate temperatures (38–42°C) it is akin to Pim\(^-\) mutants, whereas the strain displays WT properties at permissive temperatures (≤37°C).

**Induction of peroxisomes after a shift of cells from restrictive to permissive temperatures**

The reintroduction of peroxisomes in strain per13-6\(^b\) was studied after a shift of cells from restrictive to permissive growth conditions. These experiments were performed in carbon-limited continuous cultures displaying either high (growth on glucose—methanol mixtures) or low expression levels (growth on glucose alone) of AO, DHAS and CAT. After decreasing the growth temperature of a glucose—methanol-limited culture from 43°C to 35°C, peroxisomes were first observed ~1 h after the shift (Figure 2A and C). Generally one—or occasionally few—very small organelles developed which measured 0.1–0.2 μm; these newly formed organelles, invariably observed in close association with strands of ER, were located in close vicinity of the cell membrane (Figure 2A and C) and showed a crystalline substructure (Figure 2B). (Immuno)cytochemically these organelles were characterized by the presence of AO (Figure 2D), DHAS and CAT (not shown). Upon further cultivation at 35°C the newly formed organelles increased in size and number; the kinetics of peroxisomal growth and subsequent multiplication by fission were identical to those described before for WT cells during adaptation of cells to methylotrophic growth (Veenhuis et al., 1979). As indicated before, glucose—methanol-limited grown per13-6\(^b\) cells contain large cytosolic AO crystalloids at restrictive temperatures. However, these cytosolic crystalloids were not incorporated in the newly formed peroxisomes (Figure 2B) after the shift of cells to permissive growth conditions (35°C). Instead, these crystalloids remained virtually unaffected in the cytosol and still displayed AO activity (Figure 2D) after 36 h of cultivation at 35°C. After ~80 h of cultivation at 35°C, when the culture had reached a steady state, the per13-6\(^b\) cells were morphologically identical to normal WT cells, grown on methanol, in that they contained many large peroxisomes (compare with Figure 1A).

Comparable peroxisome induction patterns were observed upon a temperature shift of partly derepressed per13-6\(^b\) cells grown in a continuous culture on glucose alone. Under these conditions the cells contain moderate levels of AO and CAT, which remain soluble in the cytosol; generally, AO crystalloids are not observed (van der Klei et al., 1991a). Interestingly, also in these cells AO and CAT activities (judged from cytochemistry) remained present in the cytosol of the cells after prolonged cultivation at permissive temperatures in spite of the fact that peroxisomes were now present.

**Are cytosolic matrix and membrane proteins incorporated in newly synthesized peroxisomes induced after a shift of cells from restrictive to permissive growth conditions?**

The results presented above suggest that at least part of the cytosolic peroxisomal matrix proteins are not incorporated in newly formed peroxisomes after a shift of per13-6\(^b\) cells from restrictive to permissive growth conditions. This raises the question whether these proteins are essential to allow
the reintroduction of peroxisomes. Moreover, based on the general view that every membrane must originate from a pre-existing membrane (Borst, 1989), the question arises whether already present peroxisomal membrane proteins (PMPs) are essential for the initiation of peroxisome development under such conditions. As shown before, apart from matrix proteins PMPs are also normally synthesized in constitutive Per− mutants of H. polymorpha and are located in proteinaceous/phospholipid aggregates in the cells (Sulter et al., 1993a). In order to investigate these fundamental questions in more detail, the inducible peroxisomal matrix protein amine oxidase (AMO) and a heterologous 47 kDa peroxisomal membrane protein (PMP47) from Candida boidinii (Goodman et al., 1986; McCammon et al., 1990) were used as reporter proteins. Earlier experiments showed that PMP47 is not present in H. polymorpha but correctly sorts to peroxisomes when artificially expressed in either WT or in per13-6Δ cells grown at permissive temperatures. On the other hand, in restrictively grown per13-6Δ cells, PMP47 appeared to be located, as in constitutive Per− mutants in cytosolic aggregates, together with homologous H. polymorpha PMPs (Sulter et al., 1993b).

The per13-6Δ transformant, containing the gene encoding PMP47 from C. boidinii under control of the H. polymorpha AO promoter (PMOX; Sulter et al., 1993b), was grown in a glucose-limited continuous culture at 43°C in the presence of ammonium as nitrogen source. Under these conditions PMOX is partly derepressed (Egli et al., 1980), resulting in moderate levels of cytosolic AO and PMP47 in the cells (compare Figure 6A and B), whereas the synthesis of AMO is fully repressed (Zwart et al., 1983). Subsequently, these cells were used as inoculum for fresh batch cultures containing (i) glucose in order to repress PMOX fully, plus (ii) ethylamine to induce the synthesis of the new peroxisomal matrix enzyme AMO and incubated at 35°C to allow peroxisome development. Furthermore, to ascertain full repression of PMOX and depletion of mRNAs encoding AO and PM47, the glucose-limited cells were exposed to an excess of glucose for 30 min at 43°C prior to the shift to glucose-ethylamine-containing media. The respective locations of AO, AMO and PMP47 during the initial hours of permissive growth after the shift should provide an answer to the question whether already synthesized AO and PMP47 are included in the initially developing peroxisomes. Biochemical analysis on crude extracts of cells incubated for 4.5 h (t = 4.5) in the glucose-ethylamine-containing medium at 35°C indicated that AMO activity was indeed induced (specific activity 6.4 mU/mg protein). The locations
of AO, AMO, CAT and PMP47 were studied biochemically by conventional cell fractionation methods including differential centrifugation of homogenized protoplasts. The distributions of AO, AMO and CAT activities in the different fractions are shown in Figure 3. The data indicate that both in the inoculum cells and in cells at $t = 4.5$ h AO is present in a soluble form, whereas at $t = 4.5$ h AMO is sedimentable, suggesting a particulate nature. CAT showed a different sedimentation pattern. As expected, it was completely soluble in the inoculum cells; however, at $t = 4.5$ h part of CAT was sedimentable (Figure 3). The above enzyme distribution pattern is confirmed by Western blotting of the different fractions (Figure 4); these experiments also indicated that proteolytic degradation of neither AO nor CAT had occurred in cells incubated for 4.5 h in the new growth environment.

As expected from earlier experiments (Sulter et al., 1993b), PMP47 was sedimentable in the P3 (30 000 g fraction) obtained from cell homogenates of both the inoculum cells ($t = 0$) and cells harvested at $t = 4.5$ h (Figure 4). Therefore, differential centrifugation could not discriminate between a possible peroxisomal or aggregated nature of PMP47. However, after sodium carbonate treatment of the P3 fractions prepared from the inoculum cells grown at 43°C ($t = 0$) and from cells harvested at $t = 4.5$ h, PMP47 protein was solely detected in the soluble fraction (Figure 5). In control experiments, however, using P3 pellets of per13-6S transformants grown at permissive temperatures and of the homologous organism C. boidinii, PMP47 protein remained sedimentable after carbonate treatment (Goodman et al., 1986; Figure 5). From this we conclude that in both the inoculum cells and the cells at $t = 4.5$ h, PMP47 did not behave like an integral membrane protein.

The subcellular location of AO, AMO and PMP47 after the shift of transformed per13-6S cells to glucose—
Fig. 6. Immunocytochemical experiments, performed on PMP47-expressing transformed cells of \textit{per13-6'} grown in a glucose-limited continuous culture, which were used as inoculum for fresh glucose–ethylamine-containing batch cultures. In these cells AO is located in the cytosol (A; anti AO–protein A–gold), whereas PMP47 is located in a cytosolic aggregate (B; arrow: anti PMP47–protein A–gold). After 4.5 h of incubation in glucose–ethylamine-containing media the cells contain a small peroxisome (C), which are the sole sites of AMO protein (D; anti AMO–protein A–gold) and activity (E; CeCl$_3$ + ethylamine); in these cells AO is still in the cytosol, including the nucleus, but not in the newly developed peroxisomes (F; anti AO–protein A–gold). N, nucleus; M, mitochondrion; V, vacuole; P, peroxisome. Bars represent 0.5 μm.

ethylamine media and permissive growth conditions (35°C) was also studied by ultrastructural methods. Analysis of ultrathin sections indicated that as expected, cells of transformed \textit{per13-6'} grown in a glucose-limited continuous culture at 43°C (t = 0) lacked peroxisomes (Figure 6A) whereas at $t = 4.5$ h they generally contained a single—or a few—small organelles (Figure 6C–F). Cytochemical and immunocytochemical experiments revealed that both the location and the activity of AO at $t = 0$ (Figure 6A) and $t = 4.5$ h (Figure 6F) are confined to the cytosol (and the nucleus), whereas at $t = 4.5$ h AMO protein and activity were solely detected in the peroxisomal matrix (Figure 6D
and E). Also in these experiments the cytosolic AO remained active during prolonged cultivation of cells in the glucose–ethylamine media (Figure 7C). This clearly shows that mature, cytosolic AO is neither essential nor used for initial peroxisome development.

Immunocytochemically, PMP47 was never found associated with the newly formed peroxisomes present in the cells 4.5 h after the shift. Instead, PMP47-containing aggregates which were already present in the inoculum cells (compare with Figure 6B), still could be observed, spatially separated from peroxisomes (Figure 7A). Also at later stages of cultivation PMP47 was not detectable in the peroxisomal membrane (Figure 7B). In controls, in which the cells were transferred to methanol–ethylamine-containing media (conditions in which \(P_{\text{MOX}}\) is not repressed), PMP47 is normally sorted to the membranes bounding the new AO containing peroxisomes (inset Figure 7D). Therefore, our combined biochemical and ultrastructural data strongly suggest that both in the inoculum cells \((t = 0)\) as well as in cells incubated for 4.5 h in glucose–ethylamine-containing media, PMP47 is not located in peroxisomal membranes but solely in the cytosolic aggregates.

**Discussion**

This paper describes the isolation and characterization of ts peroxisome-deficient \((per)\) mutants of the methylotrophic yeast *H. polymorpha*. The isolation procedure was based on previous findings that *per* mutants of this organism could be identified within a collection of mutants impaired in the utilization of methanol as sole carbon source (Mut\(^-\) mutants; Cregg et al., 1990; Titorenko et al., 1993). Following the same strategy, 97 ts Mut\(^-\) mutants of *H. polymorpha* were obtained, among which two mutants were identified which showed a Per\(^+\) phenotype at restrictive temperatures (43°C), but displayed WT properties at permissive temperatures (\(<37^\circ\)C). Genetic analysis revealed that both mutant phenotypes were due to monogenic recessive mutations which mapped in the same gene, designated *PER13*.

Unexpectedly, a Pim\(^-\) phenotype (Waterham et al., 1992b) was observed at intermediate temperatures (38–42°C). This implicates that in *per13-6\(\theta\)* the presence or absence of a complete and functional organelle can be manipulated by changing the growth temperature. Apparently, the proper assembly of peroxisomes is dependent on the
amount of functional PER13 protein present at the different
temperatures. This is the first example of such a phenomenon.
It is probably unique for microbodies and only possible
because of the fact that peroxisome deficiency, unlike the
situation for other cell organelles, is not lethal in yeasts.
In fact, different peroxisomal functions may be effectively
carried out in the cytosol (e.g. nitrogen and ethanol
metabolism; Sulter et al., 1990, 1991), although growth
on methanol (in the case of \( H.\) polymorpha) or oleic acid
(in the case of \( S.\) cerevisiae) as respective
carbon sources is strictly dependent on the presence of
intact peroxisomes (van der Klei et al., 1991a; Kunau
and Hartig, 1992).

At present it is generally accepted that upon their induction
yeast microbodies develop by fission from pre-existing
organelles (Lazarow and Fujiki, 1985; Veenhuis and Harder,
1987; Borst, 1989). However, in \( per13-6\text{ts} \) cells grown at
restrictive temperatures intact peroxisomes are completely
lacking thus raising the question where the new peroxisomes,
synthesized after the shift of cells from restrictive to
permissive growth conditions, originated from. Our results
indicate that the mature, cytosolic AO present at restrictive
temperatures is not incorporated into the newly synthesized
organelles. This most probably is also true for CAT. The
synthesis of this enzyme remains induced after the shift
of the cells to the new growth environment (glucose –
ethylamine) and therefore the sedimentable part of CAT
observed after 4.5 h of incubation of the cells in permissive
growth conditions may in fact reflect CAT protein, which
is newly synthesized after the temperature shift.

Different observations were made on peroxisome-deficient
Zellweger fibroblasts; when cells from different complementation
groups were fused to heterokaryons, cytosolic catalase became incorporated into particles (suggested to
represent peroxisomes) (Brul et al., 1988). Also different
matrix proteins microinjected into human fibroblasts were
transported into peroxisomes independently from the presence
of cycloheximide (Walton et al., 1992). However, the
mechanisms involved in this process, e.g. whether mature
cytosolic matrix proteins are (partially) unfolded prior to
import, are still unknown. In our experiments on \( per13-6\text{ts} \),
however, import of mature AO protein was never observed,
despite the very high detection limit of the cytochemical
method used (Veenhuis et al., 1979). This is in line with
earlier experiments (Douma et al., 1990), which revealed
that import also did not occur after external supplementation
of peroxisomal matrix proteins by means of liposome fusion
to yeast protoplasts prepared from WT \( H.\) polymorpha.
Therefore, peroxisomal protein import machineries might
differ between lower and higher eukaryotes.

A further discrepancy observed between mammalian and
yeast peroxisome-deficient cells includes the fate of
peroxisomal membrane proteins in these mutants. In the case
of mammalian peroxisome-deficient cell lines, evidence for
so-called peroxisomal ghost structures was presented (Santos
et al., 1988a,b; Wiemer et al., 1989; Zoeller et al., 1989).
These ghosts are defined as 'empty' membrane vesicles
containing peroxisomal membrane proteins. In \( Per^- \) mutants
of \( H.\) polymorpha, however, comparable structures were
never observed; instead, peroxisomal membrane proteins
were shown to accumulate in proteinaceous/phospholipid
aggregates (Sulter et al., 1993a) which were also recognized
by an artificially expressed, heterologous peroxisomal
membrane protein (Sulter et al., 1993b). To study whether
these structures could serve as a template for the initiation
of peroxisome development in \( per13-6\text{ts} \), we used the
heterologous peroxisomal membrane protein PMP47 of
\( C.\) boidinii (Goodman et al., 1986) as a reporter membrane
protein. As shown previously, PMP47 normally sorts to
peroxisomes of both \( S.\) cerevisiae (McCammon et al., 1990)
and \( H.\) polymorpha (Sulter et al., 1993b). Our combined
morphological and biochemical results, however, provided
no evidence for any involvement of such membrane protein
aggregates in the initial development of new peroxisomes
following the shift of cells of \( per13-6\text{ts} \) to permissive growth
conditions. Instead our results strengthened the earlier
assumption that in \( H.\) polymorpha \( Per^- \) mutants peroxisomal
membrane proteins are present in proteinaceous aggregates
(Sulter et al., 1993a) rather than being incorporated in
membrane vesicles ('ghosts') since biochemically PMP47
behaved differently from properly membrane-inserted
PMP47 in WT control cells.

Allen et al. (1989) showed that restoration of peroxisome
biogenesis in peroxisome-deficient Chinese hamster ovary
(CHO) cells only occurred when the cells were fused with
both WT karyoplasts and WT cytoplasts. Their results
suggest that the presence of a cytoplasmic component (intact
peroxisomes or peroxisomal precursor) is essential for the
observed reinitiation of peroxisome biogenesis. These results
imply that apparently also in peroxisome-deficient CHO cells
the putative peroxisomal ghost structures, described for these
cells, were not sufficient to restore peroxisome biogenesis
after fusion and are therefore in line with our present
observations on \( H.\) polymorpha \( per13-6\text{ts} \). In this respect it
is relevant to mention that recently evidence was presented
that peroxisomal ghosts in Zellweger fibroblasts do not reflect
stable structures, but instead may represent degradative
autophagic lysosomes (Heikoop et al., 1992).

We have not been able to identify any peroxisomal pre-
structure or other subcellular component of \( per13-6\text{ts} \) which
served as a possible template for the reintroduction of
peroxisomes after exposure of restrictive cells to permissive
growth conditions. This unexpected result indicates that in
yeasts an alternative mode of peroxisome biogenesis may
exist in addition to the well-documented process of growth
and fission observed upon the induction of fungal micro-
organisms by certain growth substrates (Lazarow and Kindl,
1982; Veenhuis and Harder, 1987). The molecular basis
underlying this alternative mechanism is still not clear;
however, the observation that new microbodies are invariably
observed in close association with strands of ER suggest that
this compartment may play a role in this process. Studies to
e elucidate this intriguing question further are now under way.

Materials and methods

Organisms and growth conditions

\( H.\) polymorphaNCYC-495 and a \( ts \) peroxisome-deficient (\( Per^- \) ) mutant
\( T6 \) (designated \( per13-4\text{ts} \)) derived from this strain were used in all
experiments. Both the WT strain and \( per13-6\text{ts} \) were grown in continuous
medium containing 0.25% (w/v) glucose or a mixture of 0.25% (w/v)
- 0.20% (v/v) methanol in the presence of 0.20% (w/v)
ammonium sulphate as the nitrogen source at a dilution rate of 0.07 h as
described by van der Klei et al. (1991a). The WT strain was grown at 37°C,
\( per13-6\text{ts} \) at either 43 or 35°C. In order to study the proliferation of
peroxisomes as a function of the temperature, \( per13-6\text{ts} \) was also grown
at intermediate temperatures ranging from 43 to 30°C.
In addition the ts mutant was grown in batch culture in mineral medium (Veenhuis et al., 1979) and tested for growth on different carbon sources (namely glucose (0.25% w/v), ethanol and methanol, both used at 0.25% (w/v) and different nitrogen sources namely (m)ethylamine and t-alanine, all used at 0.25% (w/v)) at both the restrictive (43°C) and the permissive temperature (35°C).

Mutant isolation and genetic methods
The ts mutant of *H. polymorpha* was isolated from N-methyl-N'-nitro-N-nitrosoguanidine (NTG) followed by nystatin enrichment and subsequent isolation of methanol non-utilizing (Mut-) mutants has been described before by Cregg et al. (1990), Waterham et al. (1992b) and Titorenko et al. (1993). The temperature of 43°C was used as restrictive for enrichment and screening of ts Mut- mutants. Selected ts Mut- mutants were subsequently pregrown in batch cultures on YEPD medium, containing 1% (w/v) yeast extract, 2% (w/v) peptone and 1% (w/v) glucose at 43°C and, after two refreshments, diluted 1:4 in mineral medium containing 0.5% (v/v) methanol as the sole carbon source. After 24 h of incubation at 43°C putative Per- mutants, characterized by the presence of large cytosolic AO crystalloids, were selected by phase contrast light microscopy (van der Klei et al., 1991b; Veenhuis, 1992; see also insets in Figure 1A and B) and subsequently examined by electron microscopy.

Mating of strains, complementation analysis and random spore analysis were performed as described previously by Waterham et al. (1992b) and Titorenko et al. (1993).

Shift experiments
The fate of cytosolic peroxisomal membrane and matrix proteins, present in per1-3d- grown at the restrictive temperature following a shift of cells to permissive growth conditions, was studied in transformants of per1-3d-, expressing a heterologous peroxisomal membrane protein, namely PMP47 of *C. boidinii* (McCammom et al., 1990) under the control of RpoX, as a reporter gene. Previous experiments (Sulter et al., 1993b) showed that during growth of this transformant at restrictive temperatures, PMP47 is located in cytosolic proteinaceous/phospholipid aggregates, together with other homologous *H. polymorpha* peroxisomal membrane proteins. However, in identically transformed *W. polymorpha* PMP47 protein is correctly sorted to the peroxisomal membrane (Sulter et al., 1993b). Cells of the transformant per1-3d- were grown at the restrictive temperature (43°C) in a carbon-limited continuous culture (D = 0.075 h) on a medium containing 0.5% (w/v) glucose as carbon source and 0.2% (w/v) ammonium sulfate as nitrogen source. Subsequently, steady state cells which contained moderate levels of AO and PMP47 as a reflection of the cultivation conditions (Egli et al., 1980; Sulter et al., 1993b) were used as inoculum for fresh batch cultures containing 0.5% (w/v) glucose in the presence of 0.25% (v/v) ethylamine as the nitrogen source and further incubated at 35°C. However, in this new growth environment (i) RpoX and thus alcohol oxidase (AO) and PMP47 synthesis, is fully repressed by the excess glucose (Veenhuis et al., 1983), whereas (ii) simultaneously a new peroxisomal matrix enzyme, namely AMO, is induced by the amine substrate (Zwart et al., 1983) and (iii) peroxisome development is initiated by decreasing the growth temperature to 35°C. Prior to this shift of cells to the above batch cultures, the cells were pre-incubated for 30 min at 43°C in batch cultures containing 1% (w/v) glucose – 0.25% (w/v) ammonium sulfate in order to deplete remaining mRNAs of AO and PMP47. In addition, WT transformants were identically grown as controls.

Samples were taken from the inoculum cells pre-incubated at 43°C in glucose–ammonium sulfate and after 4.5 h of incubation in glucose–ethylamine medium and analysed for the presence of peroxisomes and location of AO, AMO, CAT and PMP47.

Preparation of crude extracts and biochemical methods
Crude extracts were prepared as described previously by Waterham et al. (1992a). AO and AMO activities were assayed by the method of Verduyn et al. (1984), CAT activity by the method of Lück (1963). Protein concentrations were determined as described by Bradford (1976) using bovine serum albumin as standard. SDS–PAGE was performed as described by Laemmli (1970). Gels were stained with Coomassie Brilliant blue R-250. Western blotting experiments were performed with the ECL Western blotting analysis system (Amersham) using specific polyclonal antibodies against AO, AMO, CAT and PMP47 (Brunner et al., 1989) and monoclonal antibodies against PMP47 (Goodman et al., 1986). Transfer of proteins onto nitrocellulose after SDS–PAGE using a semi-dry electroblotter (Ancos-Denmark) was according to Kyhse-Andersen (1984).

Cell fractionation experiments
For cell fractionation, protoplasts were prepared from variously grown cells and subsequently homogenized as described by Douma et al. (1985). The homogenate was subjected to differential centrifugation (g×min), followed by centrifugation at 12 000 g for 10 min and 30 000 g for 30 min. The 30 000 g pellet (P3) and supernatant (S3) were used for biochemical analysis.

Carbonate extraction
Carbonate extraction was essentially performed as described by McCammom et al. (1990). Aliquots of the 30 000 g (P3) pellets obtained from methanol-grown *C. boidinii*, per1-3d- grown at 30°C and per1-3d- at t = 0 and t = 4.5 h after the shift in temperature from 43°C to 35°C, were resuspended in 280 μL MES+ buffer (5 mM 2(N-morpholin)ethane sulfonic acid (pH 5.8) supplemented with 1 mM MgCl₂ and 1 mM EDTA). Sodium carbonate (final concentration 0.1 M) was added and the mixtures were incubated on ice for 1 h with occasional shaking, followed by centrifugation in a Beckman TLX Tabletop Ultracentrifuge (65 000 r.p.m., TLA-100.4 fixed angle rotor) for 1.5 h at 4°C. The resulting supernatants were saved; the pellets were resuspended in 280 μL MES+ buffer, supplemented with sodium carbonate (0.1 M final concentration). Subsequently, the supernatants and pellets were precipitated with 8.5% (v/v) trichloroacetic acid, washed twice with 80% (v/v) acetone and prepared for SDS–PAGE.

Electron microscopy
Whole cells were fixed with 1.5% (v/v) KMnO₄ for 20 min at room temperature. Spheroplasts were fixed in 6% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 60 min at 0°C, followed by postfixation in a mixture of 0.5% (v/v) OsO₄ and 2.5% (v/v) K₂Cr₂O₇ 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 a Philips EM 300. Micrographs shown are of KmO₄-fixed cells unless otherwise stated.

Cytochemistry and immunocytochemistry
Cytochemical staining experiments for the subcellular localization of AO, AMO and CAT activities were performed by the methods described previously by van Dijken et al. (1975) and Veenhuis et al. (1976). For immunocytochemistry intact cells were fixed in 3% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 90 min at 0°C, dehydrated in a graded ethanol series and embedded in Lowicryl K4M. Immunolabelling was performed on ultrathin sections with specific antibodies against AO, DHAS, AMO, CAT and PMP47 by the protein A–gold method described by Slot and Geuze (1984).

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