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# The membrane of peroxisomes in *Saccharomyces cerevisiae* is impermeable to NAD(H) and acetyl-CoA under *in vivo* conditions

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**We investigated how NADH generated during peroxisomal β-oxidation is reoxidized to $NAD^+$ and how the end product of β-oxidation, acetyl-CoA, is transported from peroxisomes to mitochondria in *Saccharomyces cerevisiae*. Disruption of the peroxisomal malate dehydrogenase 3 gene (*MDH3*) resulted in impaired β-oxidation capacity as measured in intact cells, whereas β-oxidation was perfectly normal in cell lysates. In addition, *mdh3*-disrupted cells were unable to grow on oleate whereas growth on other non-fermentable carbon sources was normal, suggesting that MDH3 is involved in the reoxidation of NADH generated during fatty acid β-oxidation rather than functioning as part of the glyoxylate cycle. To study the transport of acetyl units from peroxisomes, we disrupted the peroxisomal citrate synthase gene (*CIT2*). The lack of phenotype of the *cit2* mutant indicated the presence of an alternative pathway for transport of acetyl units, formed by the carnitine acetyltransferase protein (*YCAT*). Disruption of both the *CIT2* and *YCAT* gene blocked the β-oxidation in intact cells, but not in lysates. Our data strongly suggest that the peroxisomal membrane is impermeable to NAD(H) and acetyl-CoA *in vivo*, and predict the existence of metabolite carriers in the peroxisomal membrane to shuttle metabolites from peroxisomes to cytoplasm and vice versa.**

*Key words*: β-oxidation/carnitine acetyltransferase/citrate synthase/glyoxylate cycle/malate dehydrogenase

## Introduction

Peroxisomes are essential subcellular organelles involved in a variety of metabolic processes. Their importance is underlined by the recognition of an increasing number of inherited diseases in man in which one or more peroxisomal functions is impaired (Wanders *et al.*, 1988; Moser, 1991; Van den Bosch *et al.*, 1992).

For most of the enzymatic pathways delineated so far, peroxisomes are dependent on efficient communication with the remainder of the cell. For instance, the first two steps of the biosynthesis of ether-linked phospholipids in mammalian cells take place in peroxisomes while synthesis is completed in the endoplasmic reticulum. This involves export of the intermediate alkyl-dihydroxyacetone phosphate across the single membrane bounding the peroxisome (Van den Bosch *et al.*, 1992). A related microbody-like organelle, the glycosome of trypanosomes, contains the major part of the glycolytic pathway, implying that dihydroxyacetone phosphate, glycerol-3-phosphate, 3-phosphoglycerate and cofactors should be able to pass the glycosomal membrane (Opperdoes and Borst, 1977). However, how transfer of such metabolites across the peroxisomal membrane takes place is still a matter of debate (reviewed by Borst, 1989). One school of thought is that peroxisomes are freely permeable to low molecular weight compounds. This was concluded from the behaviour of peroxisomes upon equilibrium density gradient centrifugation in sucrose and the finding that several enzymes such as D-amino acid oxidase, glycolate oxidase and urate oxidase failed to exhibit structure-linked latency (de Duve and Baudhuin, 1966). Direct permeability measurements using patch clamp analysis provided evidence in favour of this concept (Van Veldhoven *et al.*, 1987). The other opinion holds that this permeability observed *in vitro* is a result of their isolation and that peroxisomes *in vivo* are closed compartments. This concept finds support by the observation that peroxisomes in *Hansenula polymorpha* have an acidic interior which implies restricted permeability of the peroxisomal membrane toward protons (Nicolay *et al.*, 1987; Waterham *et al.*, 1990). In addition, the observed latency of glycosomal enzymes suggests a permeability barrier for phosphorylated substrates and cofactors involved in trypanosomal glycolysis (Opperdoes and Borst, 1977).

A solution to overcome a membrane barrier is to use shuttle-systems, as in mitochondria (reviewed by Walker and Runswick, 1993). Here, transport of reducing equivalents from the cytosol to mitochondria is mediated by the glycerol-3-phosphate/dihydroxyacetone phosphate shuttle (Zebe *et al.*, 1959) or the malate/aspartate shuttle (Borst, 1963), whereas the transport of acetyl-CoA is mediated by an acetylcarnitine shuttle (Bieber, 1988). If similar shuttles are operative in peroxisomes it predicts the existence of a set of specific enzymes that participate in these exchange processes.

Here we have re-investigated the issue of peroxisome permeability using a genetic approach to study how the end products of the β-oxidation of fatty acids in *Saccharomyces cerevisiae*, acetyl-CoA and reducing equivalents (NADH), leave the peroxisome for further metabolism in the cytosol and mitochondria. Our results indicate that peroxisomes are impermeable to these compounds and that specific shuttles are required to facilitate transport across the peroxisomal membrane.

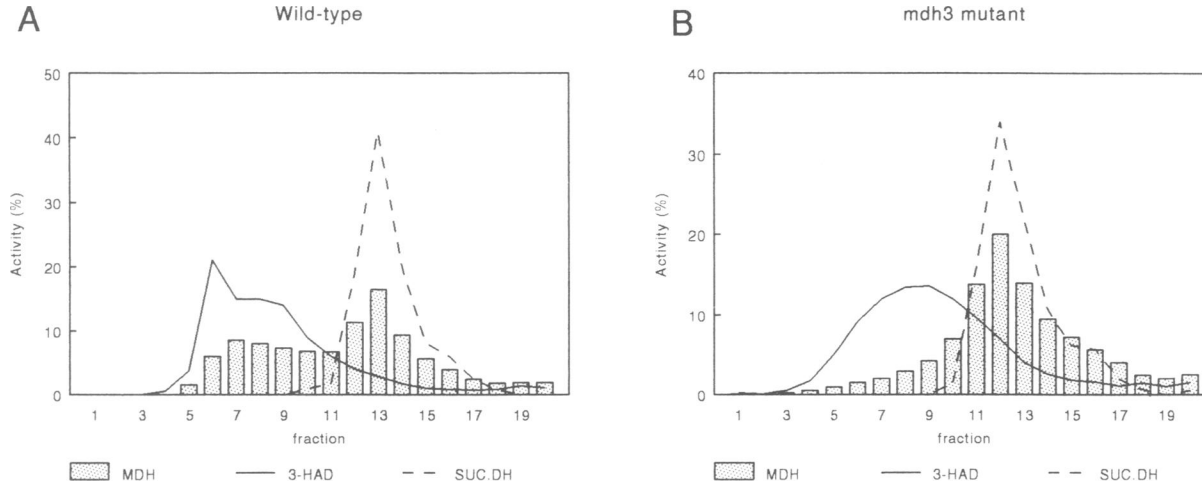


**Fig. 1.** Subcellular location of malate dehydrogenase in *S.cerevisiae*. An organellar pellet was obtained by subcellular fractionation of oleate-grown cells and used for density gradient centrifugation on Nycodenz. Fraction 1 presents the bottom fraction, fraction 20 the top fraction. Succinate dehydrogenase and 3-hydroxyacyl-CoA dehydrogenase were measured as mitochondrial and peroxisomal markers, respectively. (**A**) Gradient of wild-type cells; (**B**) gradient of Δ*mdh3* cells.

## Results

### *Malate dehydrogenase 3 is present in peroxisomes*

Transport of reducing equivalents from cytosol to mitochondria in higher eukaryotes, has long been known to be mediated by the glycerol-3-phosphate/dihydroxyacetone phosphate shuttle and the malate/aspartate shuttle. In analogy to the latter shuttle, a candidate enzyme for the reoxidation of NADH in peroxisomes of *S.cerevisiae* is the malate dehydrogenase enzyme (MDH). Earlier studies have revealed the existence of three MDH isozymes in *S.cerevisiae* (McAllister and Thompson, 1987; Minard and McAllister-Henn, 1991; Steffan and McAllister-Henn, 1992). The C-terminus of MDH3 ends in SKL (Steffan and McAllister-Henn, 1992) which is an established peroxisomal targeting signal (PTS) (Gould *et al.*, 1989). However, the presence of MDH3 in peroxisomes has not, as yet, been demonstrated.

We disrupted the *MDH3* gene and tested whether this resulted in the absence of malate dehydrogenase in the peroxisomal fractions. Therefore, cells were grown in a medium containing oleate, a well-known inducer of peroxisomes, followed by subcellular fractionation and density gradient centrifugation of the organellar pellet. The results presented in Figure 1 show good resolution between peroxisomes and mitochondria as exemplified by the distinct profiles of activity of succinate dehydrogenase (a mitochondrial marker) and 3-hydroxyacyl-CoA dehydrogenase (a peroxisomal marker). Importantly, malate dehydrogenase activity showed a bimodal distribution profile coinciding with the peroxisomal and mitochondrial fractions in wild-type cells (Figure 1A). No peroxisomal MDH activity was observed in the Δ*mdh3* cells (Figure 1B), indicating that the *MDH3* gene encodes the peroxisomal malate dehydrogenase.

If peroxisomal MDH is involved in reoxidation of intraperoxisomal NADH, one would expect induction of activity by oleate since oleate is known to induce the peroxisomal β-oxidation capacity and thus the production of NADH many-fold. Northern blot analysis indeed showed profound induction of *MDH3* mRNA by growth on oleate, whereas expression of the actin gene (used as a control) is almost constant under the various growth conditions (Figure 2). The observed induction of *MDH3* is very similar to that found for the β-oxidation enzymes.

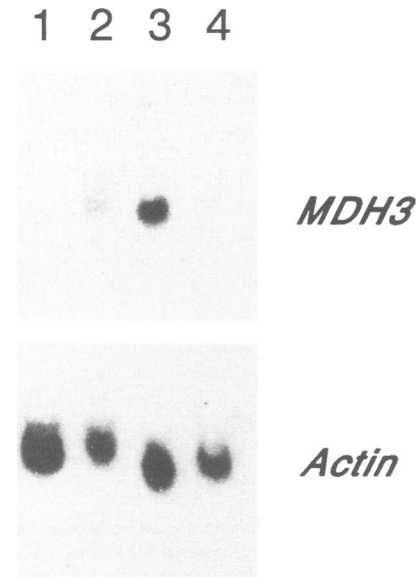


**Fig. 2.** Northern-blot analysis of *MDH3* expression. Cells were grown on medium containing glucose (1), glycerol (2), oleate (3) or acetate (4) as sole carbon source. Total RNA (10 μg) was used for each lane of the agarose gel. After blotting onto nitrocellulose, the filters were probed with the radiolabelled *MDH3* gene or with the actin gene as a control.

### *MDH3 is essential for growth on oleate*

To investigate a role for peroxisomal malate dehydrogenase in reoxidation of intraperoxisomal NADH, the growth rates of wild-type and Δ*mdh3* strains were compared on plates containing either oleate, acetate, ethanol or glycerol as the sole carbon source. Growth of Δ*mdh3* cells on acetate, ethanol or glycerol was unaffected (not

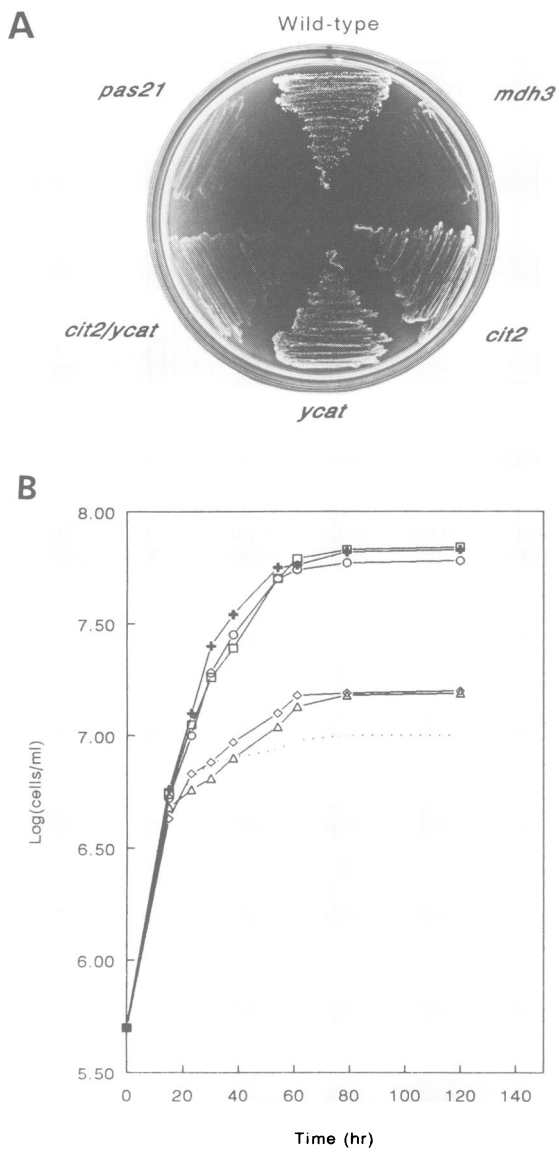


**Fig. 3.** Growth of wild-type cells and mutant cells on oleate medium. (**A**) Growth on a plate containing minimal oleate medium. The pas21 mutant (disturbed in the assembly of peroxisomes) is used as control for no growth. (**B**) Growth curves of wild-type and mutant strains on rich oleate medium. The strains shown are: wild-type cells (+), Δ*mdh3* cells (Δ), Δ*cit2* cells (○), Δ*ycat* cells (□) and Δ*cit2*/Δ*ycat* cells (◇). As a control, wild-type cells were grown on the same medium without Tween/oleate (dashed line).

shown), but growth on oleate was strongly impaired (Figure 3), suggesting that peroxisomal malate dehydrogenase is not taking part in the glyoxylate cycle, but is involved in the β-oxidation of fatty acids.

To ascertain whether the inability to grow on oleate is caused directly by a block in peroxisomal β-oxidation, we studied the oxidation of a 1-$^{14}$C-labelled fatty acid (octanoic acid) in control and Δ*mdh3* mutant cells. As shown in Figure 4, oxidation of [1-$^{14}$C]octanoic acid was strongly impaired in the intact Δ*mdh3* cells. Importantly, fatty acid oxidation was normal in Δ*mdh3* cell-free lysates in which the membrane barriers of the different intracellular organelles were absent and $NAD^+$ was present in excess. The rates of fatty acid oxidation represent the sum of [$^{14}$C]$CO_2$ and water-soluble material after extraction of the fatty acid. This gives a much better estimate of fatty acid oxidation than the amount of [$^{14}$C]$CO_2$ alone, since only part of the acetyl-CoA produced during β-oxidation is converted into $CO_2$ (Veerkamp *et al.*, 1986). The results of Figure 4 suggest that the impairment in fatty acid β-oxidation in Δ*mdh3* intact cells is caused solely by the absence of peroxisomal malate dehydrogenase, and not by reduced induction or activity of the enzymes involved directly in β-oxidation, which include acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase.

### *Accumulation of 3-hydroxyacyl-CoA intermediates in the Δmdh3 mutant*

If *in vivo* the block in β-oxidation is indeed due to the inability to reoxidize peroxisomal NADH in the absence of MDH3, this should be reflected in the accumulation of the 3-hydroxyacyl-CoA ester in the Δ*mdh3* cells but not in control cells. We tested this notion in the experiment depicted in Figure 5. Oleate-induced wild-type and Δ*mdh3* cells were incubated for 0, 30 or 60 min with radiolabelled fatty acid. The various labelled acyl-CoA esters, including the acyl-CoA ester itself and the α,β-unsaturated, 3-hydroxy- and 3-ketoacyl-CoA esters, were extracted and separated on thin layer plates. The results show that significant levels of the 3-hydroxyacyl-CoA ester were found only in Δ*mdh3* cells incubated with radiolabelled fatty acid for 30 or 60 min (Figure 5). The observed accumulation of the 3-hydroxyacyl-CoA intermediate in the Δ*mdh3* mutant suggests again that MDH3 participates in a redox shuttle rather than in the glyoxylate cycle.

### *Removal of acetyl-CoA from peroxisomes*

Acetyl-CoA is the end product of the β-oxidation of straight-chain fatty acids in yeast. An important function of the glyoxylate cycle is the condensation of two C2 units (acetyl-CoA) to succinate, thereby enabling the cell to form C4 carbon skeletons from C2 units. In addition, if peroxisomes are impermeable for (acetyl-) CoA, this cycle is also important for releasing the CoA for continuing cycles of β-oxidation and for facilitating the transport of carbon units across the peroxisomal membrane.

To test whether peroxisomes are indeed impermeable for acetyl-CoA, we tested the effect on β-oxidation by disrupting the peroxisomal citrate synthase (*CIT2*) gene (Lewin *et al.*, 1990). Growth on oleate and oxidation of [1-$^{14}$C]octanoic acid were investigated as described above for the Δ*mdh3* mutant. The results depicted in Figure 6 (second bar) show that oxidation of octanoate was normal in cells deficient in peroxisomal citrate synthase. Moreover, growth on oleate was indistinguishable from wild-type cells (Figure 3). However, since growth of Δ*cit2* cells is almost normal on ethanol and acetate as well (not shown), these results strongly suggest the existence of an efficient bypass that can compensate for the loss of peroxisomal citrate synthase.

One possible bypass route would be the conversion of acetyl-CoA into acetylcarnitine via carnitine acetyltransferase (CAT) which is known to be present in mitochondria and peroxisomes in higher eukaryotes and yeast (Markwell *et al.*, 1973, 1976; Markwell and Bieber, 1976; Kawamoto *et al.*, 1978; accompanying paper, Elgersma *et al.*, 1995). The acetylcarnitine formed in peroxisomes might sub-

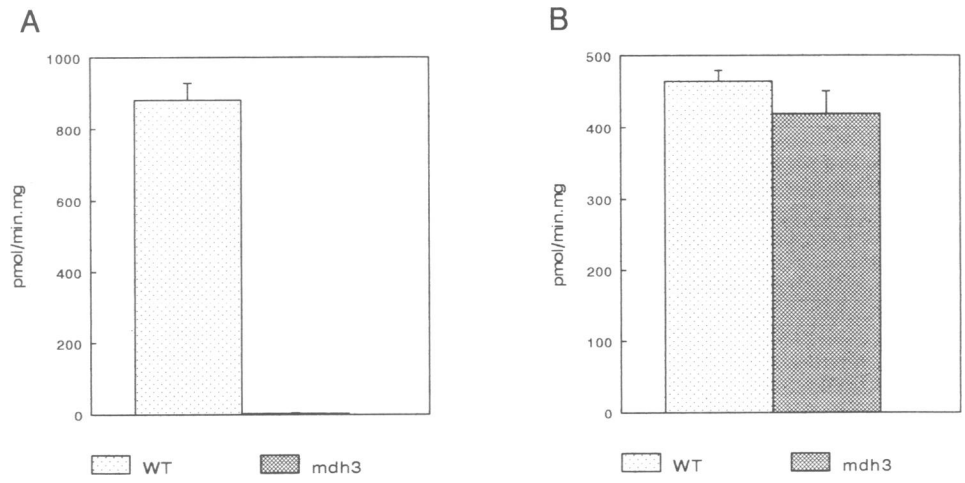


**Fig. 4.** Octanoic acid β-oxidation in oleate-induced wild-type cells and Δ*mdh3* cells. (**A**) β-oxidation in intact cells; (**B**) β-oxidation in cell lysates. [1-$^{14}$C]octanoic acid oxidation is expressed as the sum of [$^{14}$C]$CO_2$ and water-soluble β-oxidation products produced.

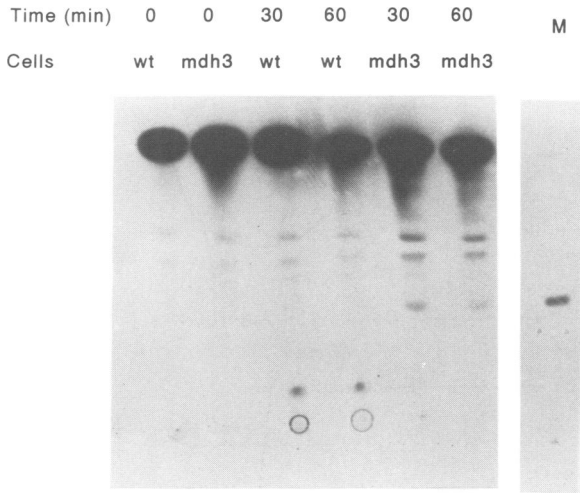


**Fig. 5.** TLC analysis of the $^{14}$C-labelled products derived from fatty acid oxidation in wild-type cells and Δ*mdh3* cells. The products formed were analysed after incubating cells 0, 30 and 60 min with [1-$^{14}$C]palmitate. The right-hand panel shows a marker lane of [1-$^{14}$C]3-hydroxypalmitoyl CoA.

sequently be transported to mitochondria where it could be further oxidized to $CO_2$ in the Krebs cycle. However, in the absence of the CIT2 protein, the citrate (or isocitrate) formed in mitochondria may also be retrieved for net synthesis of carbon skeletons in the glyoxylate cycle, as depicted in Figure 7. The possibility that peroxisomal and mitochondrial citrate synthase may be able to take over each other's function has earlier been suggested for *S.cerevisiae* based on gene deletion studies (Kispal *et al.*, 1988), and for the ethylamine-grown yeast *Trichosporon cutaneum* based on the enzymatic contents of peroxisomes (Veenhuis *et al.*, 1986).

Elgersma *et al.* (1995) demonstrated in the accompanying paper that carnitine acetyltransferase is indeed present in peroxisomes and mitochondria of oleate-grown *S.cerevisiae*. Both enzymes are encoded by the same gene, *YCAT*. Figure 6 shows that deletion of the *YCAT* gene results in a slight decrease in [1-$^{14}$C]octanoic acid oxidation in intact cells (Figure 6A, third bar) and in cell lysates (Figure 6B, third bar). However, the growth rate on oleate appeared not to be affected (Figure 3). Furthermore, growth on ethanol and acetate was normal (not shown). However, when we disrupted both the *CIT2* gene and the *YCAT* gene, the cells could no longer grow on oleate (Figure 3). Since the Δ*cit2/ycat* cells were also unable to grow on ethanol and acetate (not shown) we conclude that the YCAT protein is indeed indispensable for the net synthesis of C4 carbon units in case the *CIT2* gene is deleted.

Since the *YCAT* gene disruption results in the absence of both the peroxisomal and mitochondrial carnitine acetyltransferase protein (Elgersma *et al.*, 1995) the observed inability of Δ*cit2/ycat* cells to grow on oleate may be caused exclusively by the impermeability of mitochondria towards acetyl-CoA. To investigate directly the permeability properties of the peroxisomal membrane towards acetyl-CoA we investigated the ability of the Δ*cit2/ycat* double mutant to β-oxidize fatty acids as described above for the Δ*mdh3* mutant. Indeed, similar to what we found for the Δ*mdh3* mutant, the β-oxidation was practically blocked in intact Δ*cit2/ycat* mutant cells, whereas the β-oxidation capacity was hardly affected in cell lysates in which the membrane barriers are lost (compare Figure 6A with B, fourth bar). This strongly suggests that there are only two pathways for the export of acetyl-CoA from peroxisomes; either via conversion into glyoxylate cycle intermediates or via conversion into acetylcarnitine. Consequently, blocking both pathways leads to the accumulation of peroxisomal acetyl-CoA, which depletes the peroxisomes from free CoA for continued β-oxidation or inhibits the β-oxidation enzymes by product inhibition (Hovik and Osmundsen, 1989).

## Discussion

We have used a novel approach to obtain information on the permeability properties of peroxisomes in the yeast *S.cerevisiae*. This is based on disruption of specific genes encoding proteins involved in the preparatory steps for the transport of metabolites generated from fatty acid β-oxidation across the peroxisomal membrane. We first concentrated on peroxisomal malate dehydrogenase (MDH3) which was recently cloned and sequenced by the group of McAllister-Henn (Steffan and McAllister-Henn,

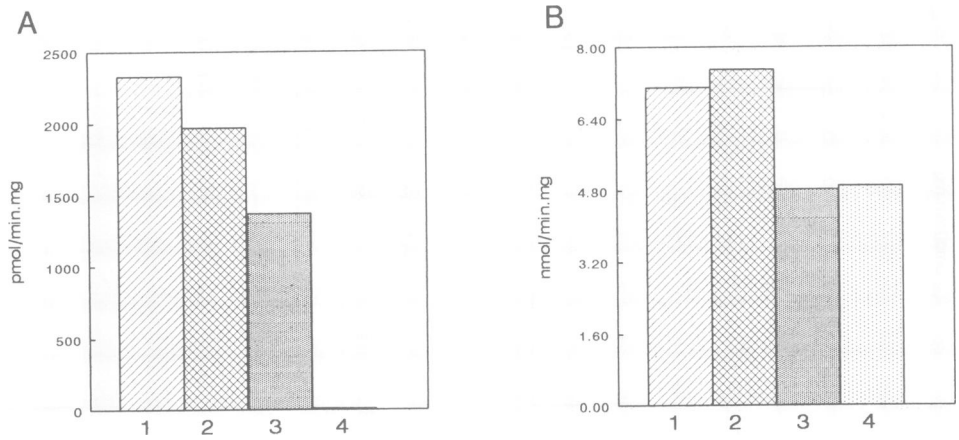


**Fig. 6.** Octanoic acid β-oxidation in oleate-induced wild-type and mutant cells. (**A**) β-oxidation in intact cells; (**B**) β-oxidation in cell lysates. [1-$^{14}$C]octanoic acid oxidation was measured as the sum of [$^{14}$C]$CO_2$ and water-soluble material produced. The bars represent wild-type cells (1), Δ*cit2* cells (2), Δ*ycat* cells (3) and Δ*cit2*/Δ*ycat* cells (4).

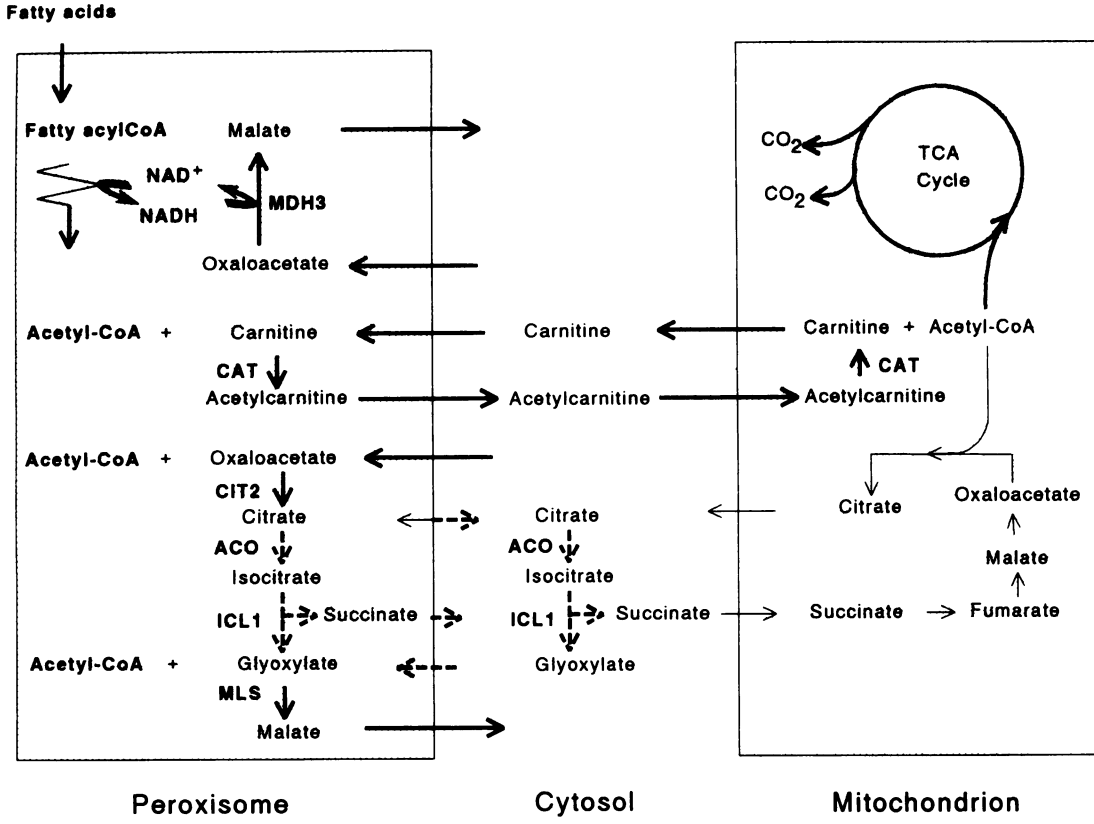


**Fig. 7.** Model for the reoxidation of intraperoxisomal NADH and the pathways for the transport of acetyl-CoA. Our results do not rule out that other metabolites than malate and oxaloacetate are shuttled between peroxisome and cytosol. Since the presence of aconitase and isocitrate lyase in peroxisomes has not been demonstrated unambiguously, these reactions might also take place in the cytosol (bold–dashed arrows). The thin arrows indicate the supposed retrieval pathway of mitochondrial citrate or isocitrate for use in the glyoxylate cycle in case the *CIT2* gene has been deleted. MDH3, malate dehydrogenase 3; CAT, carnitine acetyltransferase; CIT2, peroxisomal citrate synthase; ACO, aconitase; ICL, isocitrate lyase; MLS, malate synthase.

1992). Earlier studies (McAllister and Thompson, 1987; Minard and McAllister-Henn, 1991) had identified a mitochondrial (MDH1) and cytosolic malate dehydrogenase (MDH2). Based on the presence of a serine–leucine–lysine (SKL) tripeptide at the C-terminus, the MDH3 isoenzyme was speculated to be located in peroxisomes (Steffan and McAllister-Henn, 1992). Our results indicate that this is indeed the case. Disruption of the *MDH3* gene led to the total absence of MDH activity in peroxisomes, and to complete impairment of growth on medium containing oleate as the sole carbon source due to a peroxisomal β-oxidation deficiency. In cell lysates where reoxidation of $NAD^+$ is not required since this cofactor is present in the reaction medium, fatty acid oxidation was completely normal. These data strongly suggest that the peroxisomal membrane is impermeable to NAD(H) *in vivo*, and that malate dehydrogenase is involved in regeneration of intraperoxisomal $NAD^+$. In

such a shuttle mechanism malate dehydrogenase catalyses the reduction of oxaloacetate to malate with concomitant formation of $NAD^+$ from NADH, followed by the shuttling of malate versus oxaloacetate across the peroxisomal membrane. We cannot exclude other shuttles, for example, a malate/aspartate shuttle system. Such a shuttle would require the presence of aspartate aminotransferase protein in peroxisomes, which converts aspartate into oxaloacetate. We are currently investigating whether this protein is indeed present in peroxisomes of *S.cerevisiae*.

The finding that Δ*mdh3* cells are not impaired in growth on C2 carbon sources suggests that this enzyme does not participate in the glyoxylate cycle. Moreover, the enzyme would then have to operate in two directions in the same compartment, which is obviously impossible. Indications that the kinetic parameters of glyoxysomal malate dehydrogenase are unfavourable to its participation in the glyoxylate cycle of plant glyoxysomes were earlier reported by Mettler and Beevers (1980). The consequence of these findings is that malate produced by the glyoxylate cycle is transported out of the peroxisome followed by retroconversion to oxaloacetate in the cytosol (via MDH2) or the mitochondria (via MDH1) (Figure 7).

The results described in this paper also provide new information on the way in which acetyl-CoA is transported from the interior of the peroxisome to mitochondria for further metabolism in the Krebs cycle. It was a surprise to observe that disrupting the *CIT2* gene for peroxisomal citrate synthase did not lead to a deficiency to grow on oleate or C2 compounds such as acetate or ethanol. Since assimilation of C2 compounds requires a functional glyoxylate cycle, this suggests that citrate or isocitrate from mitochondria can reach the cytoplasm (or even the peroxisomes) as has been proposed earlier (Veenhuis *et al.*, 1986; Kispal *et al.*, 1988). Our results indicate that the carnitine acetyltransferase protein is essential for this bypass (Figure 7).

The finding that intact cells with either the *CIT2* or the *YCAT* gene disruption have almost normal capacity to oxidize fatty acids whereas β-oxidation is blocked when both genes are disrupted, indicates that there are only two ways in which acetyl-CoA can leave the peroxisome: via conversion into glyoxylate cycle intermediates or via conversion into acetylcarnitine by carnitine acetyltransferase. Consequently, since β-oxidation is virtually normal in cell-free lysates of Δ*cit2/ycat* cells (where the membrane barriers are absent), we conclude that acetyl-CoA (or CoA itself) cannot freely pass the peroxisomal membrane.

It has been notoriously difficult to establish whether all glyoxylate cycle enzymes are located in peroxisomes of *S.cerevisiae*. The presence of isocitrate lyase and aconitase in peroxisomes is still a matter of debate, as indicated in Figure 7. However, these uncertainties do not compromise the implication we propose here with regard to the impermeability of the peroxisomal membrane to NAD(H) and acetyl-CoA. The enzymes of the β-oxidation pathway that produce these compounds and the enzymes that take care of the preparatory steps in removal of these compounds from peroxisomes (MDH3, CIT2, MLS1 and YCAT) are all localized in peroxisomes beyond any doubt. One of the predictions of our results is that, in analogy with mitochondria, the peroxisomal membrane must contain a variety of different transport-proteins such as carnitine/acetylcarnitine carriers and dicarboxylate carriers.

## Materials and methods

### *Yeast strains and culture conditions*

All the gene disruptions used for this study were made in the *S.cerevisiae* strain BJ1991 (*MATα, leu2, trp1, ura3–251, prb1–1122, pep4–3*). Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO)(DIFCO), 2% glucose and amino acids (20 μg/ml) as needed. The liquid media used for growing cells for RNA isolations, growth curves or subcellular fractionation contained 0.5% potassium phosphate buffer, pH 6.0, 0.3% yeast extract, 0.5% peptone, and 2% glucose, or 2% glycerol, or 2% K-acetate, or 0.12% oleic acid/0.2% Tween-40 as carbon source. Before shifting to one of these media, cells were grown on minimal 0.3% glucose medium for at least 24 h. For RNA isolations, the cultures were inoculated at such a density that they reached $OD_{600}$ = 0.7–1.0 after ~15 h. Oleic acid plates contained 0.1% oleic acid/0.4% Tween-40, 0.67% yeast nitrogen base without amino acids (YNB-WO)(DIFCO), 0.1% yeast extract (DIFCO) and amino acids (20 μg/ml) as needed.

### *Cloning procedures*

Standard DNA techniques were carried out as described (Sambrook *et al.*, 1989). The yeast *MDH3* gene was amplified using two oligonucleotide primers corresponding to regions of non-homology with *MDH1* and *MDH2*. Oligonucleotide sequence from the 5′ end of the gene was (5′-TTTGAATTCAAGCATAAAACAATCAAGG-3′). The oligonucleotide sequence from the 3′ end of the gene was (5′-GGATCCGATATGAGT-CAAGATACAAAGG-3′). A *S.cerevisiae* genomic DNA library was used as a template in a polymerase chain reaction (PCR) using the above two primers. The PCR reaction was carried out using 0.5 μg of template DNA, 167 μg/ml oligonucleotides, 10 mM dNTPs, 0.5 units of Taq polymerase (Boehringer Mannheim), 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 8.4, 50 mM KCl and 0.01% BSA in a total volume of 50 μl. The annealing temperature was 55°C. A 1.2 kb *Eco*RI–*Bam*HI fragment was obtained and subcloned into the multiple cloning site of pUC19. The *Eco*RV–*Nco*I fragment containing 600 bp of the *MDH3* open reading frame was replaced using blunt ended cloning, with a 2.2 kb fragment of the *LEU2* gene. Linearized plasmid DNA with the disrupted *MDH3* gene was used to transform a haploid wild-type strain BJ1991. $Leu^+$ transformants were confirmed for disruption of the chromosomal *MDH3* locus by analysing the PCR product obtained on chromosomal DNA with the same primers as used earlier to amplify the gene.

The *CIT2* gene deletion was made by isolating genomic DNA of PSY42-*cit2* cells (*Leu2-2, leu2-112, lys2-801, CIT2::URA3*) (kindly provided by A.Shyan and R.Butow). This was used as template for PCR with the *CIT2* primers (5′-GGATCCATGACAGTTCCTTATCTA-3′) and (5′-CTATAGTTTGCTTTCAATGTT-3′). The resulting PCR fragment was used to transform BJ1991 cells, and $ura^+$ transformants were selected for integration in the *CIT2* gene by PCR analysis.

The *YCAT* gene was amplified from genomic DNA using the 5′ CAT-A primer (5′-TTTGAATTCGAGAACTCTCTCAAAC-3′) and the 3′ CAT-B primer (5′-TTTCTGCAGCGTAAGCCCTTTTTTCTCCC-3′) oligonucleotides. The annealing temperature used for the PCR reaction was 55°C. The resulting 2.1 kb *Eco*RI–*Pst*I fragment was subcloned into pUC19 (pEL72). The major part of the open reading frame was deleted, by replacing the *Acc*1–*Bgl*2 fragment (containing 1281 bp of the CAT open reading frame) by the LEU2 gene (pEL78). This plasmid was used to transform wild-type cells (BJ1991) and Δ*cit2* (PSY142) cells. $Leu^+$ transformants were selected for integration in the *YCAT* gene by PCR analysis.

### *Subcellular fractionation and Nycodenz gradients*

Subcellular fractionations were performed as described (Van der Leij *et al.*, 1992). Organellar pellets were used for continuous 16–35% Nycodenz gradients (12 ml), with a cushion of 1 ml 42% Nycodenz dissolved in 5 mM MES, pH 6.0, 1 mM EDTA, 1 mM KCl and 8.5% sucrose. The sealed tubes were centrifuged for 2.5 h in a vertical rotor (MSE 8×35) at 19 000 r.p.m. (29 000 *g*) at 4°C.

### *β-oxidation measurements*

Oleate-grown cells were washed with water and resuspended in phosphate-buffered saline (PBS), to $OD_{600}$ = 2.5. Aliquots of 20 μl of cell suspension were used for fatty acid β-oxidation measurements in 200 μl

medium containing PBS plus 10 μM [1-$^{14}$C]palmitate or [1-$^{14}$C]-octanoate. Reactions were allowed to proceed for 6 or 12 min at 30°C, followed by termination of reactions by adding 100 μl of 1.3 M perchloric acid. Radiolabelled $CO_2$ was trapped overnight in 500 μl of 2 M NaOH. The $^{14}$C-labelled β-oxidation products were subsequently collected after extracting the acidified material with chloroform/methanol/heptane as described before (Heikoop *et al.*, 1990) and quantified in a liquid scintillation counter. Fatty acid β-oxidation activities were also measured in cell-free lysates prepared by lysing protoplasts in a medium containing 0.1% Triton X-100, 5 mM MOPS, pH 7.4, 1 mM EDTA and 1 mM PMSF. The cell-free extract was subsequently incubated in reaction medium containing the following components: 150 mM Tris–HCl, pH 8.5, 5 mM ATP, 5 mM $MgCl_2$, 2 mM NaCN, 100 μM FAD, 1 mM NAD, 1 mM CoASH, 0.005% (w/v) Triton X-100, 1 mU/ml acyl-CoA synthetase (Boehringer Mannheim) and 10 μM [1-$^{14}$C]palmitate or [1-$^{14}$C]octanoate. Reactions were allowed to proceed for 6 or 12 min, followed by quantification of [$^{14}$C]$CO_2$ and $^{14}$C-labelled β-oxidation products as described above.

### *Identification of acyl-CoA intermediates*

In order to identify the nature of the acyl-CoA esters accumulating in mutant cells, oleate-induced intact cells were incubated with 10 μM [1-$^{14}$C]palmitate as described above, for 30 or 60 min. Reactions were terminated by 100 μl 1.3 M perchloric acid. In order to hydrolyse all CoA-esters 100 μl of 2 M NaOH was subsequently added and the mixture was incubated at 50°C for 30 min. This was followed by addition of 10 μl of 0.5 M $H_2SO_4$ and 75 μl of sodium acetate buffer, pH 6.0. If required, pH was adjusted to 4.0. The fatty acids were then extracted with methanol/chloroform/heptane as described above. The lower layer was collected, washed and taken to dryness under $N_2$. The residue was taken up in acetone, followed by chromatography on TLC using conditions as described by Bremer and Wojtczak (1972), with the exception that benzene was replaced by toluene. After running, the plate was dried and subjected to autoradiography. The recovery of fatty acid derivatives during extraction and solvent evaporation, acetone solubilization and TLC chromatography were checked by determining the recovery of [1-$^{14}$C]palmitoyl-CoA and enzymatically synthesized enoyl-CoA esters and 3-hydroxyacyl-CoA esters prepared from [1-$^{14}$C]palmitoyl-CoA. Recoveries were >95%.

Radioactively labelled [1-$^{14}$C]3-hydroxypalmitoyl CoA was synthesized enzymatically by incubating 10 μM [1-$^{14}$C]palmitate for 10 min with 50 U/ml crotonase (Sigma Co., St Louis, USA) and 10 U/ml acyl-CoA oxidase (Sigma) in a reaction medium as described above. Further handling of the samples was as described above for the identification of acyl-CoA intermediates.

### *Enzyme assays*

Malate dehydrogenase activity was measured as the oxaloacetate-dependent rate of NADH oxidation ($A_{340}$ nm) in assay mixtures containing 45 mM $K_3PO_4$, pH 7.4, 0.12 mM NADH, and 0.33 mM oxaloacetate (Steffan and McAllister-Henn, 1992). 3-hydroxyacyl-CoA dehydrogenase activities were measured on a Cobas–Fara centrifugal analyser by following the 3-keto-octanoyl-CoA-dependent rate of NADH consumption at 340 nm (Wanders *et al.*, 1990). Succinate dehydrogenase was measured according to a recently described method (Munujos *et al.*, 1993). Protein concentrations were determined by the bicinchoninic acid method (Smith *et al.*, 1985).

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