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Peroxisomal β-oxidation of polyunsaturated fatty acids in *Saccharomyces cerevisiae*: isocitrate dehydrogenase provides NADPH for reduction of double bonds at even positions

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The β-oxidation of saturated fatty acids in *Saccharomyces cerevisiae* is confined exclusively to the peroxisomal compartment of the cell. Processing of mono- and polyunsaturated fatty acids with the double bond at an even position requires, in addition to the basic β-oxidation machinery, the contribution of the NADPH-dependent enzyme 2,4-dienoyl-CoA reductase. Here we show by biochemical cell fractionation studies that this enzyme is a typical constituent of peroxisomes. As a consequence, the β-oxidation of mono- and polyunsaturated fatty acids with double bonds at even positions requires stoichiometric amounts of intraperoxisomal NADPH. We suggest that NADP-dependent isocitrate dehydrogenase isoenzymes function in an NADP redox shuttle across the peroxisomal membrane to keep intraperoxisomal NADP reduced. This is based on the finding of a third NADP-dependent isocitrate dehydrogenase isoenzyme, Idp3p, next to the already known mitochondrial and cytosolic isoenzymes, which turned out to be present in the peroxisomal matrix. Our proposal is strongly supported by the observation that peroxisomal Idp3p is essential for growth on the unsaturated fatty acids arachidonic, linoleic and petroselinic acid, which require 2,4-dienoyl-CoA reductase activity. On the other hand, growth on oleate which does not require 2,4-dienoyl-CoA reductase, and NADPH is completely normal in Δ*idp3* cells.

Keywords: 2,4-dienoyl-CoA reductase/isocitrate dehydrogenase/β-oxidation/polyunsaturated fatty acids/redox shuttle

Introduction

Peroxisomes are essential subcellular organelles involved in a variety of metabolic processes. Their importance is underlined by the identification of an increasing number of inherited diseases in man in which one or more peroxisomal functions are impaired (Moser, 1991; Van den Bosch *et al.*, 1992; Wanders *et al.*, 1995).

One of the main functions of peroxisomes is the degradation of fatty acids. In vertebrate cells, this takes place not only in peroxisomes but also in mitochondria. Long-chain fatty acids are oxidized in mitochondria whereas very-long-chain fatty acids are shortened in peroxisomes and oxidized to completion in mitochondria. In principle, β-oxidation in mitochondria and peroxisomes proceeds via the same mechanism, involving sequential steps of dehydrogenation, hydratation, a second dehydrogenation and thiolytic cleavage. In the case of the oxidation of mono- and polyunsaturated fatty acids, auxiliary enzyme activities are required to remove the double bonds. NADPH-dependent 2,4-dienoyl-CoA reductases (EC 1.3.1.34) and the Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerases (EC 5.3.3.8) play an essential role in the removal of double bonds. Indeed, it is now clear that Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase activity is involved in the removal of double bonds at uneven positions whereas an NADPH-dependent 2,4-dienoyl-CoA reductase is required to remove double bonds at even positions (Hiltunen, 1991; Osmundsen *et al.*, 1991; Schulz, 1991; Kunau *et al.*, 1995).

In yeast, fatty acid β-oxidation is restricted to peroxisomes (Kunau *et al.*, 1988). The fact that yeasts like *Saccharomyces cerevisiae* and *Candida tropicalis* are able to grow on different types of fatty acids including saturated and monounsaturated fatty acids (Hettema *et al.*, 1996) and polyunsaturated fatty acids (Dommes *et al.*, 1983), implies that *S.cerevisiae* and *C.tropicalis* have the capacity to remove double bonds in fatty acids.

On the basis of the *Escherichia coli* amino acid sequence of NADPH-dependent 2,4-dienoyl-CoA reductase, we have identified a reading frame in the *S.cerevisiae* database showing high amino acid similarity with the *E.coli* enzyme. The encoded *S.cerevisiae* protein contains a C-terminal peroxisomal targeting signal (PTS1). We have now found that peroxisomes of *S.cerevisiae* indeed contain NADPH-dependent 2,4-dienoyl-CoA reductase activity. In addition, we discovered a new NADP-dependent isocitrate dehydrogenase isoenzyme, which is confined to peroxisomes. Based on the finding that cells lacking this peroxisomal enzyme fail to oxidize mono- and polyunsaturated fatty acids with double bonds at the even position, we propose that the cytosolic and peroxisomal NADP-dependent isocitrate dehydrogenases function in a redox shuttle to replenish NADPH consumed in the dienoyl-CoA reductase reaction required for β-oxidation of polyunsaturated fatty acids. The implications of this work with respect to the recent demonstration of the impermeability of the peroxisomal membrane for small molecules will be discussed.

Results

Growth of S.cerevisiae on unsaturated fatty acids as sole carbon source

A favourite carbon source to induce peroxisome proliferation in *S.cerevisiae* is oleate, a monounsaturated fatty

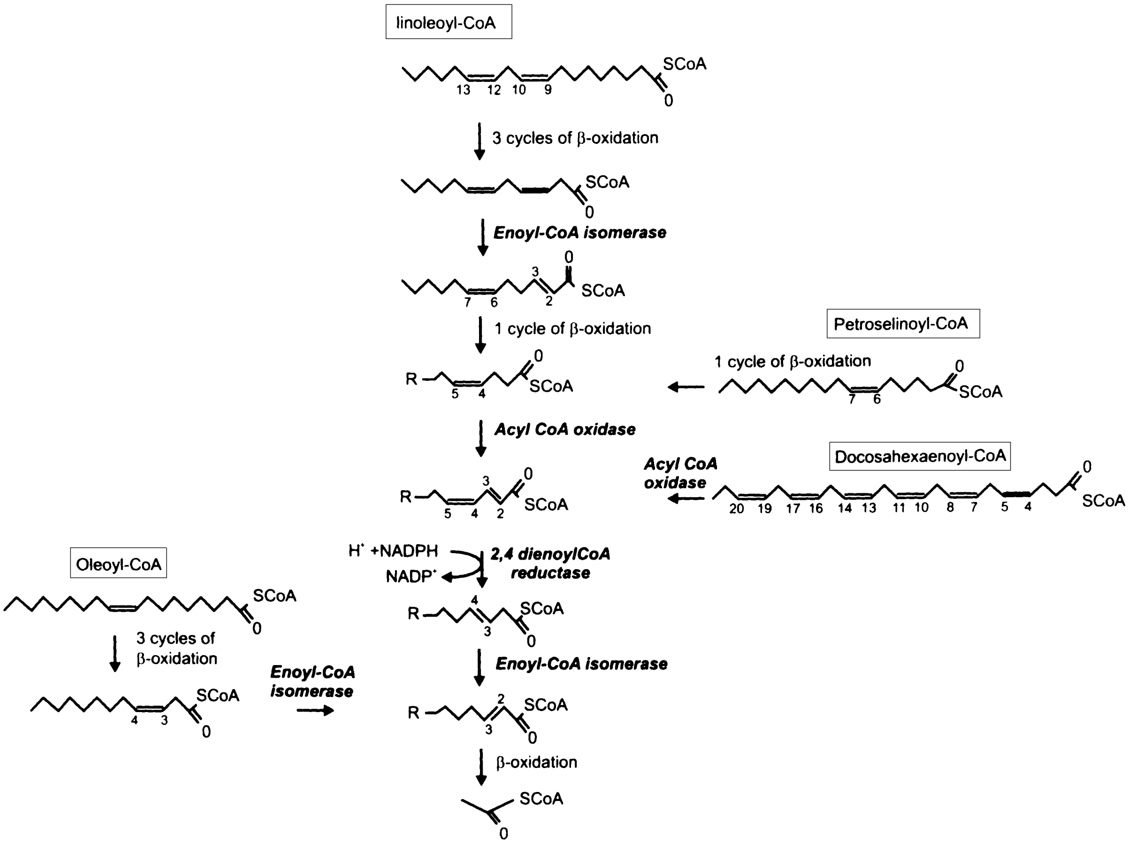

Fig. 1. Overview of β-oxidation of oleoyl-CoA (C18:1), petroselinoyl-CoA (C18:1), linoleoyl-CoA (C18:2) and docosahexaenoyl-CoA (C22:6) in *S.cerevisiae* (modified after Schulz, 1991). The basic enzymatic steps of β-oxidation (dehydrogenation/oxidation, hydratation, dehydrogenation, Δ^3-*cis*-Δ^2-*trans*- enoyl-CoA isomerase and thiolytic cleavage) are sufficient for the oxidation of saturated fatty acids and monounsaturated fatty acids with the double bond at the uneven position. An additional enzyme is required for mono- and polyunsaturated fatty acids with the double bond at the even position to prepare them for β-oxidation: NADPH-dependent 2,4-dienoyl-CoA reductase.

acid with the single double bond at the 9th position. Oleate can be oxidized by the general β-oxidation machinery comprised of acyl-CoA oxidase, multifunctional protein (2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase and 3-hydroxyacyl-CoA epimerase), 3-ketoacyl-CoA thiolase and Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase (Figure 1). An additional enzyme is required for the β-oxidation of mono- and polyunsaturated fatty acids with the double bond at an even position: NADPH-dependent 2,4-dienoyl-CoA reductase (Figure 1). To study whether *S.cerevisiae* contains NADPH-dependent 2,4-dienoyl-CoA reductase activity, we have tested whether *S.cerevisiae* could use various kinds of fatty acids for growth. Indeed *S.cerevisiae* was able to grow on mono- and polyunsaturated fatty acids with double bonds at even positions, such as (6) petroselinic acid (C18:1), (9,12) linoleic acid (C18:2) and (5,8,11,14) arachidonic acid (C20:4), implying the presence of NADPH-dependent 2,4-dienoyl-CoA reductase activity (Figure 1 and experiment not shown).

NADPH-dependent 2,4-dienoyl-CoA reductase is a peroxisomal enzyme

To determine the subcellular localization of NADPH-dependent 2,4-dienoyl-CoA reductase in *S.cerevisiae,* we measured the β-oxidation of oleic and docosahexaenoic acid (C22:6) (Figure 2) using an organellar fraction prepared by differential centrifugation of a cell-free homogenate. To this end, ^{14}C-radiolabelled oleate and docosahexaenoic acid were incubated with the organellar fraction in the presence or absence of NADPH. Oleate was degraded efficiently in the absence of NADPH while β-oxidation of docosahexaenoic acid was almost fully dependent on the presence of NADPH (Figure 2A). Virtually all docosahexaenoic acid oxidation activity was located in the organellar fraction prepared from wild-type cells (not shown). Subsequent studies showed that all 2,4-dienoyl-CoA reductase activity was present in the organellar pellet fraction (Figure 2B). Fractionation of the organellar pellet fraction by density gradient centrifugation showed that 2,4-dienoyl-CoA reductase activity was found at the density characteristic of peroxisomes (Figure 2C). Upon fractionation of a homogenate prepared from Δ*pex5* mutant cells, deficient in the PTS1 receptor, virtually all reductase activity was present in the supernatant fraction. This mislocalization suggests that NADPH-dependent 2,4-dienoyl-CoA reductase is a PTS1-containing protein.

Therefore, we conclude that peroxisomes contain NADPH-dependent 2,4-dienoyl-CoA reductase activity, which implies that intraperoxisomal NADPH is required for the function of the reductase.

NADP-specific isocitrate dehydrogenases

Previously we have shown that the peroxisomal membrane is impermeable to small molecules such as NAD(H) (Van Roermund *et al.*, 1995). The β-oxidation of petroselinic acid and docosahexaenoic acid requires stoichiometric

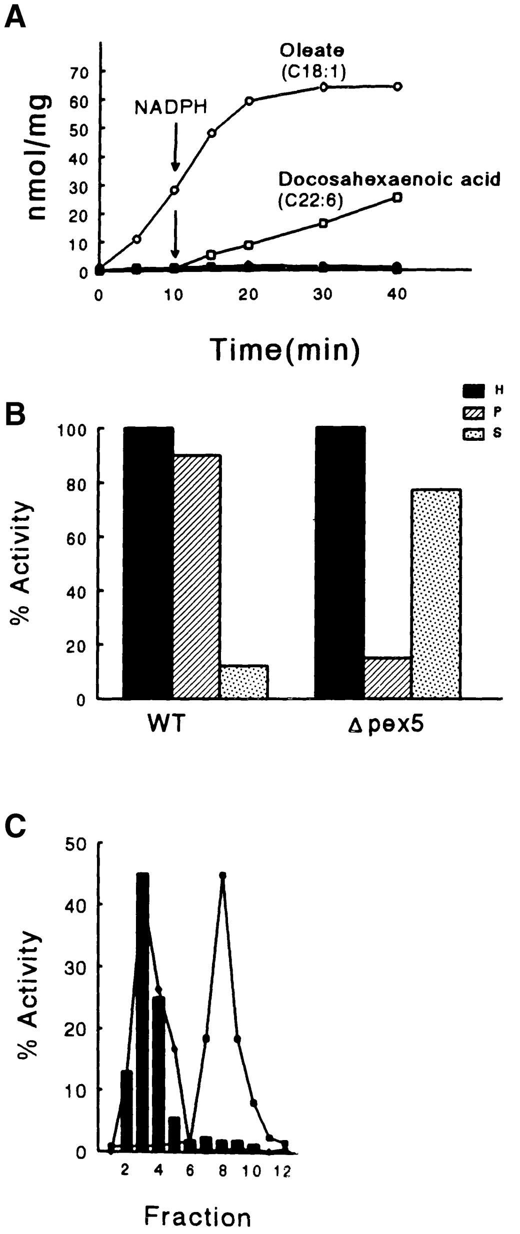

Fig. 2. Localization of NADPH-dependent 2,4-dienoyl-CoA reductase activity in peroxisomes and its involvement in docosahexaenoic acid β-oxidation (22:6) in *S.cerevisiae.* (**A**) β-oxidation of oleic (○) and docosahexaenoic acid (■ □ ●) using an organellar pellet fraction prepared by differential centrifugation of wild-type (○ □ ●) or Δ*fox1* (■) (disturbed in the acyl-CoA oxidase) cell free homogenates (see Materials and methods) and without NADPH (●). (**B**) Subcellular distribution of the NADPH-dependent 2,4-dienoyl-CoA reductase in oleate-induced wild-type and Δ*pex5* (deficient in the PTS1 receptor) cells. After centifugation, the homogenates (H), pellets (P) and supernatants (S) were assayed for activity. (**C**) Nycodenz density gradient of the organellar pellet from wild-type cells. Fraction 1 corresponds to the bottom fraction, while fraction 12 reflects the top fraction. Succinate dehydrogenase (SucDH) (■) (mitochondrial marker), 3-hydroxyacyl-CoA dehydrogenase (3-HAD) (●) (peroxisomal marker) and NADPH-dependent 2,4-dienoyl-CoA reductase (black bars) were measured in the fractions.

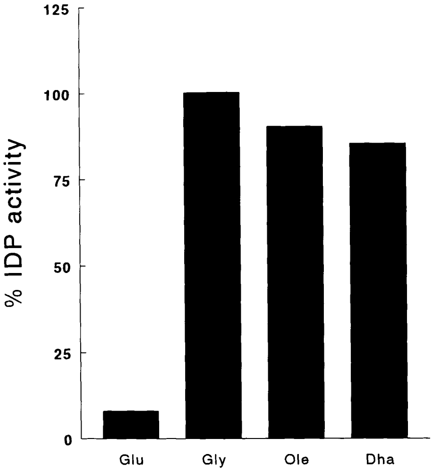

Fig. 3. NADP-dependent isocitrate dehydrogenase activity was measured in total homogenates of cells grown on either glucose (Glu)-, glycerol (Gly)-, oleate (Ole)- or docosahexaenoic acid (Dha)-containing media.

amounts of NADPH (Figure 1). This raises the question of by which mechanism NADP is reduced to NADPH. The most plausible explanation, based on analogy with transport processes across the mitochondrial inner membrane, is that the peroxisomal membrane contains transporters which are involved in a redox shuttling process. The demonstration that rat liver peroxisomes contain NADP-dependent isocitrate dehydrogenase focused our attention on this group of cellular isoenzymes (Leighton *et al.*, 1968).

NADP-dependent isocitrate dehydrogenase activity is induced by growth on fatty acids

Yeast was grown on glucose (repression of peroxisomes), glycerol (derepression of peroxisomes) or oleate (induction of peroxisomes), and total NADP-dependent isocitrate dehydrogenase activity was measured. Growth on three carbon sources, including docosahexaenoic acid (C22:6), led to enhanced levels of activity compared with glucose-grown cells (Figure 3).

To date, two genes coding for NADP-dependent isocitrate dehydrogenase enzymes have been reported in *S.cerevisiae*. The first one, originally described by Haselbeck and McAllister-Henn (1990), encodes mitochondrial Idp1p, whereas the second one codes for cytosolic Idp2p (Loftus *et al.*, 1994; Zhao and McAllister-Henn, 1996). To make an inventory of genes that are strongly expressed by growing yeast on oleate as sole carbon source, we applied serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995; Kal, 1997). Among the tags that were encountered frequently, one was derived from YNL009w, a gene with high homology to *IDP1* and *IDP2*, known as YDL066w and YLR174w in the yeast genome database, here called *IDP3*. Amino acid sequence comparison revealed strong similarity, with the exception of the amino- and carboxy-terminal ends (Figure 4). Idp1p has an N-terminal extension which functions as a mitochondrial targeting signal (MTS) (Haselbeck and McAllister-Henn, 1990). Idp3p lacks this pre-sequence but has nine additional amino acids at the C-terminus. The last three amino acids, CKL, comprise a putative PTS1 conforming to the PTS1 consensus motif established for *S.cerevisiae* (Elgersma *et al.*, 1996). Cytoplasmic Idp2p lacks both the MTS and PTS1 motifs (Figure 4).

Inspection of the 5′-regions upstream of the *IDP1, 2* and *3* genes revealed the presence of putative oleate

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      1                                                              60
Idp1p MSMLSRRLFS TSRLAAFSKI KVKQPVVELD GDEMTRIIWD KIKKKFTLIL PYLDVDLKYY
Idp2p M~~~~~~~~~ ~~~~~~~TKI KVANPIVEMD GDEQTRIIWS FNQDK..LVL PYLDVDLKYY
Idp3p M~~~~~~~~~ ~~~~~~~SKI KVVHPIVEMD GDEQTRVIWK LIKEK..LIL PYLDVDLKYY

      61                                                            120
Idp1p DLSVESRDAT SDKITQDAAE AIKKYGVGIK CATITPDEAR VKEFNLFTHK MWKSPNGTIR
Idp2p DLSVEYRDQT NDQVTVDSAT ATLKYRVAVK CATITPDEAR VEEFHL..KK MWKSPNGTIR
Idp3p DLSIQERDRT NDQVTKDSSY ATLKYGVAVK CATITPDEAR MKEFNL..KE MWKSPNGTIR

      121                                                           180
Idp1p NILGGTVFRE PIVIPRIPRL VPRWEKPIII GRHAHGDQYK ATDTLIPFTG PGSLELVYKP
Idp2p NILGGTVFRE PIIIPRIPRL VPQWEKPIII GRHAFGDQYK ATDVIVP..E EGELRLVYKS
Idp3p NILGGTVFRE PIIIPKIPRL VPHWEKPIII GRHAFGDQYR ATDIKIK..K AGKLRLQFSS

      181                                                           240
Idp1p SDPTTAQPQT LKVYDY.KGS GVAMAMYNTD ESIEGFAHSS FKLAIDKKLF TNLFLSTKNT
Idp2p KSGT..HDVD LKVFDYPEHG GVAMMMYNNT DSIEGFAKAS FELAIERKL. .PLYSTTKNT
Idp3p DDGK..ENID LKVYEFPKSG GIAMAMFNTN DSIKGFAKAS FELALKRKL. .PLFFTTKNT

      241                                                           300
Idp1p ILKKYDGRFK DIFQEVYEAQ YKSKFEQLGI HYEHRLIDDM VAQMIKSKGG FTFIMALKNY
Idp2p ILKKYDGKFK DVFEAMYLEV IKRSLESLGI WYEHRLIDDM VAQMLKSKGG ..YIMAMKNY
Idp3p ILKNYDNQFK QIFDNLFDKE YKEKFQALKI TYEHRLIDDM VAQMLKSKGG ..FIIAMKNY

      301                                                           360
Idp1p DGDVQSDIVA QGFGSLGLMT SILVTPDGKT FESEAAHGTV TRHYRKYQKG EFTETSTNSI
Idp2p DGDVESDIVA QGFGSLGLMT SVLITPDGKT FESDRAHGTV TRHLTDYDKG R..ETSTNSI
Idp3p DGDVQSDIVA QGFGSLGLMT SILITPDGKT FESEAAHGTV TRHFRKHQRG E..ETSTNSI
      361                                                           420
Idp1p ASIFAWSRGL LKRGELDNTP ALCKFANILE SATLNTVQQD GIMTKDLALA CGFTNNERSA
Idp2p ASIFAWTRGI IQRGKLDNTP DVVKFGQILE SATVNTVQED GIMTKDLALI LG..KSERSA
Idp3p ASIFAWTRAI IQRGKLDNTD DVIKFGNLLE KATLDTVQVG GKMTKDLALM LG..KTNRSS

      421                             452
Idp1p YVTTEEFLDA VEKRLQKEIK SIE~~~~~~~ ~~
Idp2p YVTTEEFIDA VESRLKKEFE AAA~~~~~~~ ~~
Idp3p YVTTEEFIDE VAKRLQNMML SSNEDKKGMC KL
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Fig. 4. Alignment of the mitochondrial (Idp1p), cytosolic (Idp2p) and peroxisomal NADP-dependent isocitrate dehydrogenase (Idp3p) amino acid sequences. Idp1p has an N-terminal mitochondrial targeting signal (MTS). Idp3p contains a nine-amino-acid C-terminal extension with a type 1 peroxisomal targeting signal (PTS1).

response elements (OREs) both in *IDP2* and in *IDP3*, but not in *IDP1*. These OREs are found in a number of oleate-inducible yeast genes including the genes coding for the β-oxidation enzymes, suggesting that cytosolic and peroxisomal isocitrate dehydrogenase may be functionally linked to fatty acid β-oxidation.

Carbon source-dependent regulation of IDP2 and IDP3 genes

Expression of genes coding for a variety of different peroxisomal proteins is dependent on the carbon source. Glucose strongly represses transcription, whereas non-fermentable sources like glycerol and ethanol derepress transcription. In addition, fatty acids strongly induce transcription of genes encoding peroxisomal proteins. We analysed the transcriptional regulation of the *IDP2* and *IDP3* genes using the luciferase reporter gene driven by the *IDP2* or *IDP3* promoter. Wild-type cells were transformed with the reporter constructs and cultured on glucose, glycerol or oleate media. Cell extracts were assayed for luciferase activity. The results (Figure 5) showed that expression of both *IDP2* and *IDP3* genes was repressed by glucose. The *IDP2* gene was strongly induced by both glycerol and oleate. The *IDP3* gene was derepressed by glycerol and fully induced by oleate, which resembles the expression of other genes coding for peroxisomal β-oxidation enzymes.

Oleate induction is exerted via the transcription factors Pip2p and Oaf1p which bind as a heterodimer to the ORE in promotors of genes encoding peroxisomal proteins (Luo *et al.*, 1996; Rottensteiner *et al.*, 1996). Analyses of both the *IDP2* and *IDP3* promoter reporter constructs revealed that regulation of *IDP2* expression occurs independently of Pip2p, in contrast to *IDP3* expression. These experiments illustrate that expression of *IDP3* parallels that of other β-oxidation enzymes whereas expression of *IDP2* is regulated by an alternative mechanism.

NADP-dependent isocitrate dehydrogenase activity is present in peroxisomes, mitochondria and cytosol

To study the subcellular localization of the NADP-dependent isocitrate dehydrogenase activity in *S.cerevisiae*, a homogenate of oleate-grown cells was first subjected to differential centrifugation (Figure 6A). Most of the activity was found in the cytosolic fraction. The organellar fraction was fractionated further by density gradient centrifugation on Nycodenz. Figure 6B shows good separation of peroxisomes and mitochondria as monitored by the distribution of 3-hydroxyacyl-CoA dehydrogenase (peroxisomes) and succinate dehydrogenase (mitochondria). A bimodal activity profile was found for isocitrate dehydrogenase, with activity in peroxisomes and mitochondria, although

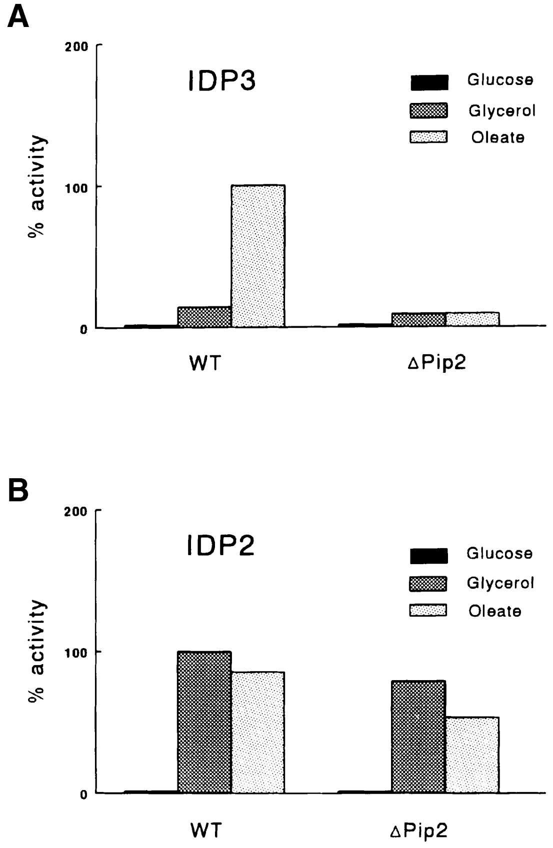

Fig. 5. Idp3p expression is regulated in parallel with peroxisomal β-oxidation enzymes. Wild-type and Δ*pip2* cells were transformed with the reporter constructs, cultured on glucose, glycerol and oleate and assayed for luciferase activity. Expression of the *IDP3* gene was dependent on Pip2p while oleate induction of the *IDP2* gene was independent of Pip2p.

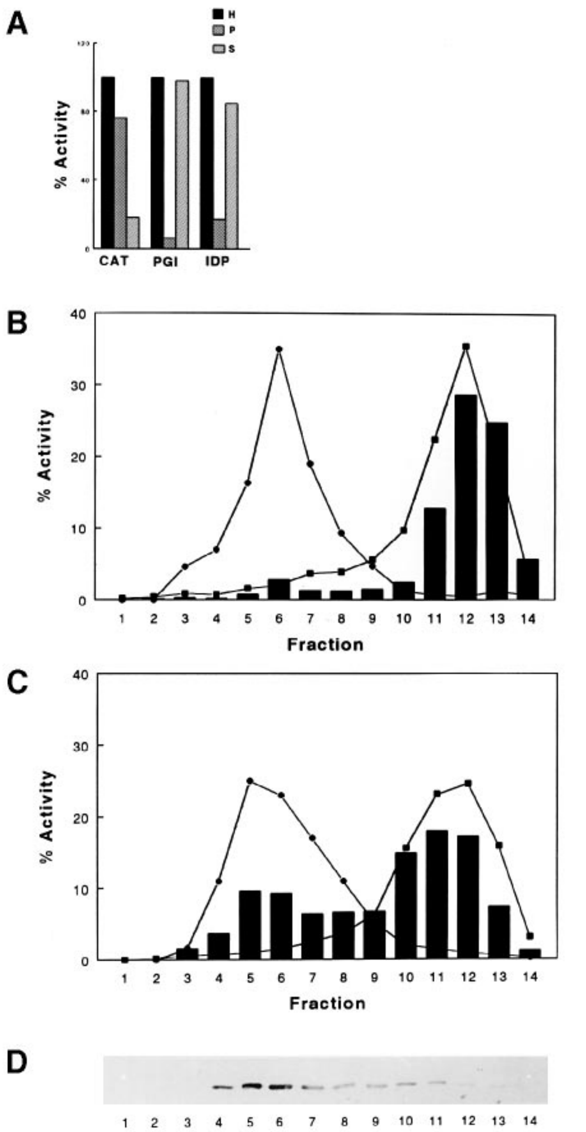

Fig. 6. Subcellular localization of Idp3p in oleate-grown cells of *S.cerevisiae*. (**A**) Subcellular fractionation of wild-type cells. Comparable volumes of homogenate (H), pellet (P) and supernatant (S) were used for measurements of catalase (CAT)-, phosphoglucose isomerase (PGI)- and NADP-dependent isocitrate dehydrogenase (IDP) activities. Percentage activity is the relative amount of activity in each fraction compared with the total amount of activity, which was present in the homogenate. Nycodenz density gradient of the organellar pellet obtained from wild-type cells (**B**) or wild-type cells transformed with the NH-Idp3p construct (**C**) (expressed under control of the *CTA1* promoter). Fraction 1 corresponds to the bottom fraction, while fraction 14 reflects the top fraction. Succinate dehydrogenase (SucDH) (■), used as mitochondrial marker, 3-hydroxyacyl-CoA dehydrogenase (3-HAD) (●), used as peroxisomal marker, and isocitrate dehydrogenase (IDP) (black bars) were measured in the fractions. NH-tagged Idp3p (**D**) was detected by immunoblotting using the NH antibody. The expressed constructs were under the control of the *CTA1* promoter. More than 90% of the NH-tagged version of Idp3p was found to be present in peroxisomes.

most isocitrate dehydrogenase activity was found to be present in the mitochondrial fractions.

Further evidence for the presence of isocitrate dehydrogenase activity in peroxisomes came from experiments in which we studied the subcellular localization of an enzymatically active NH-tagged version of Idp3p. Figure 6C shows that ~25% of the total isocitrate dehydrogenase activity in control cells transformed with a construct expressing NH-Idp3p is present in the peroxisomal fractions. In addition, immunoblot analysis showed (Figure 6D) that >90% of the NH-tagged version of Idp3p is present in the peroxisomal fractions. Furthermore, disruption of the *IDP3* gene resulted in a deficiency of the peroxisomal NADP-dependent isocitrate dehydrogenase. Taken together, these results indicate that NADP-dependent isocitrate dehydrogenase activity is located in three different compartments of the cell: cytoplasm, mitochondria and peroxisomes, and that the peroxisomal NADP-dependent isocitrate dehydrogenase activity is due to Idp3p.

Idp3p is a peroxisomal matrix protein and its import is PTS1 dependent

To confirm the presence of Idp3p inside peroxisomes, we performed immunoelectron microscopy of oleate-induced cells expressing the NH-tagged version of Idp3p from a single copy plasmid. Figure 7A shows clear labelling of the peroxisomal matrix. In the same experiment, we used NH-Idp2p as a cytosolic control (Figure 7B). More than 95% of the Idp3p gold particles were found to be present in peroxisomes and most of the labelling of Idp2p gold particles was localized in the cytosol and the nucleus.

The tagged protein also enabled us to study which

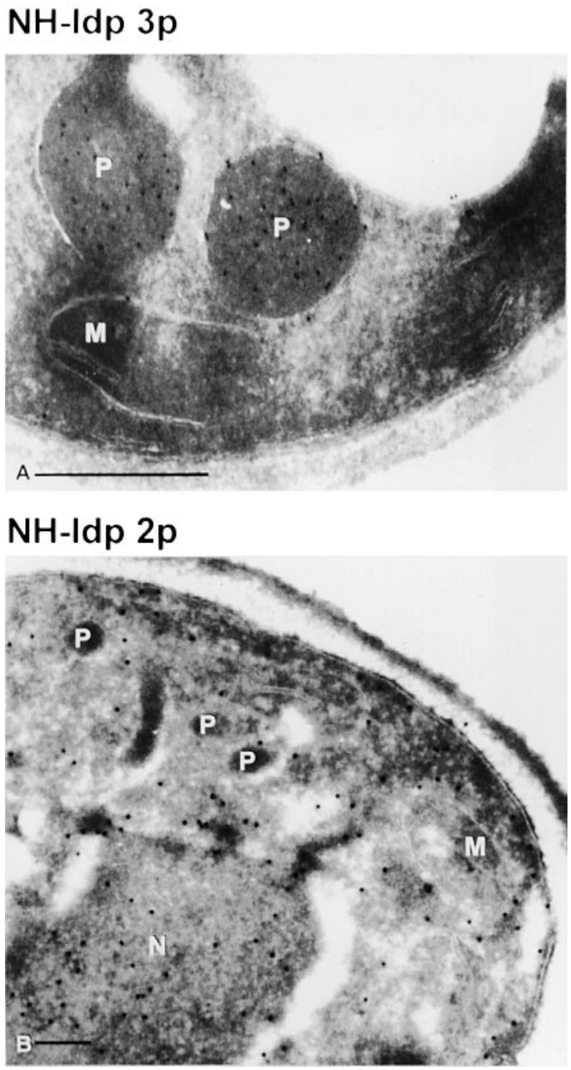

Fig. 7. Electron microscopical analysis of wild-type cells expressing (**A**) NH-Idp3p and (**B**) NH-Idp2p. Cryosections of cells grown on oleate were labelled using the NH antiserum and immunogold particles conjugated with protein A. (P) Peroxisome; (M) mitochondria; (N) nucleus. Bar = 0.2 μm.

import pathway is followed by Idp3p. For this purpose, we used two mutants with a differential defect in protein import at the level of either the PTS1 (Δ*pex5* mutant) or PTS2 (Δ*pex7* mutant) receptor (Van der Leij *et al.*, 1993; Marzioch *et al.*, 1994; Zhang *et al.*, 1995). Import of NH-Idp3p was normal in Δ*pex7* cells, but blocked in Δ*pex5* cells (Figure 8). These results indicate that the import of NH-Idp3p protein into peroxisomes is mediated via the PTS1 import pathway as expected on the basis of the predicted PTS1 (see Figure 4).

The IDP3 gene is essential for growth on arachidonic, linoleic and petroselinic acid, but not oleic acid

In order to investigate the presumed role of peroxisomal Idp3p in the reduction of NADP to NADPH within peroxisomes, growth rates of wild-type and Δ*idp3* cells were compared on various media (Figure 9).

Growth of Δ*idp3* cells on glucose, acetate, glycerol or oleate was unaffected (not shown). However, growth on linoleic and petroselinic acid was strongly impaired. These results suggest that peroxisomal NADP-dependent isocitrate dehydrogenase is not required for oleate β-oxidation, but is required for β-oxidation of arachidonic, linoleic and petroselinic acid. Importantly, oxidation of the latter three fatty acids requires the active participation of 2,4-dienoyl-CoA reductase in contrast to the β-oxidation of oleic acid.

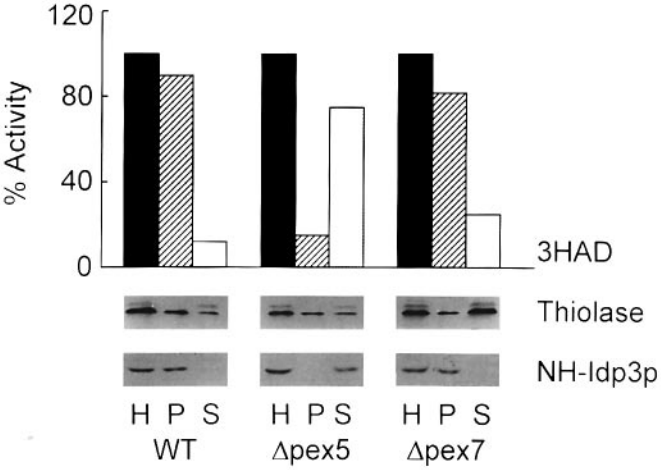

Fig. 8. Import of NH-Idp3p-, PTS-1 (3HAD)- and PTS2 (3-ketoacyl-CoA thiolase)-containing proteins in wild-type, Δ*pex5* and Δ*pex7* cells. Subcellular fractionations of wild-type, Δ*pex5* and Δ*pex7* cells expressing NH-Idp3p grown on oleate medium. The 30 000 *g* pellet fraction (P) represents the organellar fraction, whereas the supernatant (S) fraction represents the cytosolic fraction. (H) represents the homogenate before the high-speed spin. Comparable volumes were layered in every lane. NH-Idp3p and thiolase were detected by Western blot analysis. 3-hydroxyacyl-CoA dehydrogenase (3HAD) activity was measured as described in Materials and methods.

To ascertain whether the growth defect on mono- or polyunsaturated fatty acids with double bonds at even positions really resulted from an impaired degradation of these fatty acids, we studied the oxidation of [1-^{14}C]docosahexaenoic acid (C22:6) in wild-type and Δ*idp3* cells. As shown in Figure 10A, oxidation of [1-^{14}C](4,7,10,13,16,19) docosahexaenoic acid was strongly impaired in the Δ*idp3* cells but rescued after introduction of NH-tagged Idp3p (Figure 10A, lane 3). Importantly, docosahexaenoic acid (C22:6) β-oxidation was normal in the organellar fraction of Δ*idp3* cells in which the membrane barriers of the different intracellular organelles were disrupted by addition of detergent and NAD and NADPH were added in excess (Figure 10B). Figure 10 suggests that the impaired β-oxidation of polyunsaturated fatty acids is caused by the absence of peroxisomal NADP-dependent isocitrate dehydrogenase, and not by reduced induction or activity of enzymes directly involved in polyunsaturated fatty acid β-oxidation, comprising acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, Δ^3-*cis*-Δ^2-*trans*-enoyl-CoA isomerase and 2,4-dienoyl-CoA reductase.

Accumulation of (2,4,7,10,13,16,19) dienoyl-CoA intermediates in Δidp3 cells

If the block in β-oxidation of (4,7,10,13,16,19) docosahexaenoic acid (C22:6) is indeed caused by the inability to reduce peroxisomal NADP to NADPH as a result of

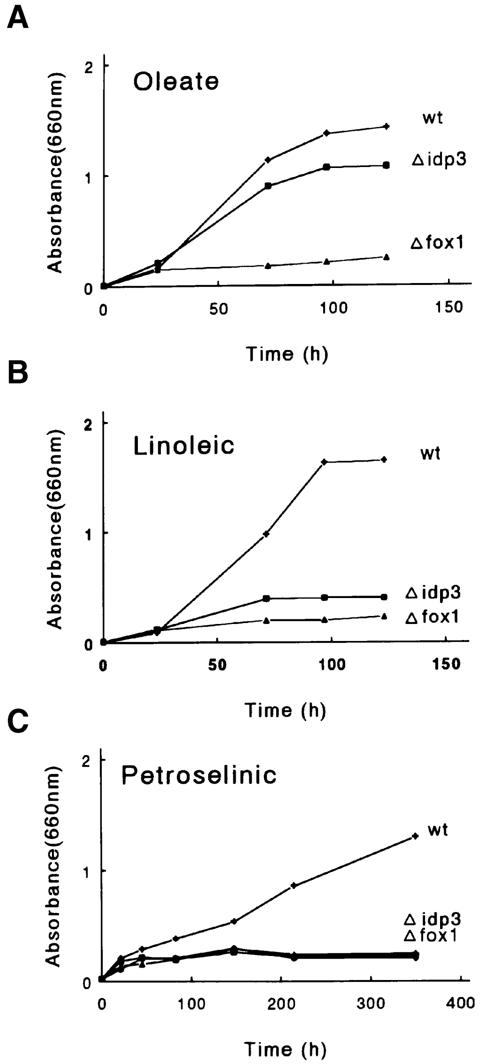

Fig. 9. Growth of wild-type and mutant cells on oleate (**A**), linoleic acid (**B**) and petroselinic acid (**C**). The strains shown are: wild-type cells (wt), Δ*idp3* cells and Δ*fox1* cells (disturbed in the acyl-CoA oxidase).

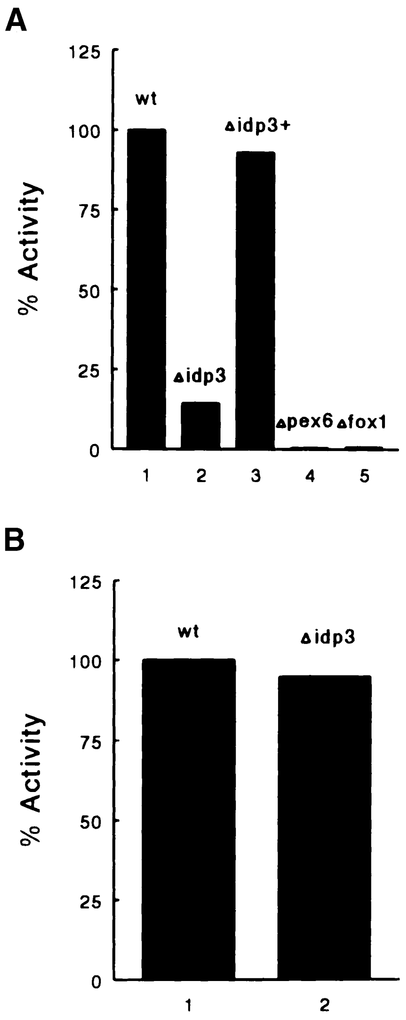

Fig. 10. β-oxidation of docosahexaenoic acid in oleate-induced wild-type and mutant cells. (**A**) Docosahexaenoic acid β-oxidation in oleate-induced wild-type cells (wt), Δ*idp3* cells and Δ*idp3*$^{+}$ cells which are transformed with an NH-IDP3 construct expressed under the control of the *CTA1* promoter, and as negative controls Δ*pex6* cells (disturbed in the assembly of peroxisomes) and Δ*fox1* cells (disturbed in the acyl-CoA oxidase). (**B**) Docosahexaenoic acid β-oxidation in an organellar pellet fraction of wild-type (wt) and Δ*idp3* cells in which the membrane barriers of the different intracellular organelles were absent and NAD and NADPH were added in excess (see Materials and methods).

the absence of Idp3p, this should be reflected in the accumulation of the substrate of the 2,4 dienoyl-CoA reductase reaction, i.e. (2,4,7,10,13,16,19) dienoyl-CoA ester (C22:7) in the Δ*idp3* cells, but not in wild-type cells (Figure 1). We tested this in the experiment depicted in Figure 11. Oleate-induced wild-type and Δ*idp3* cells were incubated for 30 and 60 min with ^{14}C-radiolabelled fatty acid. Labelled acyl-CoA esters were extracted and separated on thin-layer plates. The results show oxidation of docosahexaenoic acid (C22:6) in wild-type cells whereas oxidation is impaired in the Δ*idp3* cells. Furthermore, there was accumulation of (2,4,7,10,13,16,19) docosaheptaenoic acid (C22:7), the dehydrogenation product of docosahexaenoic acid as catalysed by acyl-CoA oxidase, in Δ*idp3* cells but not in wild-type cells. The identity of the C22:7 compound was verified by enzymatic synthesis of C22:7-CoA from C22:6-CoA (lane M). The observed accumulation of C22:7 intermediate in Δ*idp3* cells supports our hypothesis that Idp3p provides the NADPH required for the reductase step inside the peroxisomes (Schulz *et al.*, 1991). The nature of the additional band observed in Δ*idp3* cells at 30 and 60 min incubation is presently unknown.

Discussion

β-oxidation of fatty acids in mammalian cells is dependent on a complex enzymatic machinery that is divided over two cellular compartments: mitochondria and peroxisomes, with the cytoplasm functioning as an intermediate between them. This complexity arises from the structural variety of different fatty acids encountered in nature and in the cell and from the requirement for efficient communication and cross-regulation between mitochondria and peroxisomes. The presence of β-oxidation in two different organelles is reflected in the structural and enzymatic differences of the enzymes involved. For instance, the first oxidation step is catalysed by a dehydro-

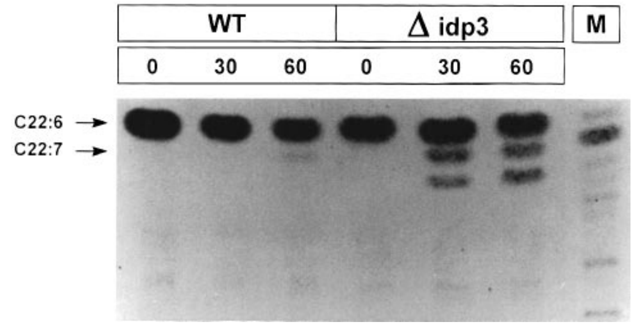

Fig. 11. Accumulation of 2,4-dienoyl-CoA ester (C22:7) during β-oxidation of docosahexaenoic acid (C22:6). TLC analysis of ^{14}C-labelled product derived from docosahexaenoic acid (C22:6) β-oxidation in wild-type (wt) and Δ*idp3* cells. The products formed were analysed after incubation for 0, 30 and 60 min with docosahexaenoic acid (C22:6). Lane M corresponds to an incubation to which acyl-CoA oxidase was added to allow conversion of C22:6-CoA to C22:7-CoA.

genase (acyl-CoA dehydrogenase) in mitochondria and by an oxidase (acyl-CoA oxidase) in peroxisomes. Moreover, in mitochondria, most of the enzymes of the basic β-oxidation machinery occur in multiple isoforms each with their own substrate specificity towards particular chain length or branching of the fatty acids (Matsuo and Strauss, 1994). Recent studies have shown the existence of a similar multiplicity in mammalian peroxisomes as exemplified by the presence of different acyl-CoA oxidases (Reddy and Mannaerts, 1994), different multifunctional enzymes (Novikov *et al.*, 1994; Dienaide-Noubhani *et al.*, 1996; Jiang *et al.*, 1996; Leenders *et al.*, 1996; Qin *et al.*, 1997) and different thiolases (Seedorf *et al.*, 1994).

Although the basic enzymatic steps of β-oxidation (dehydrogenation/oxidation, hydration, a second dehydrogenation, Δ^3-*cis*-Δ^2-*trans*- enoyl-CoA isomerase and the thiolytic cleavage) are sufficient for the oxidation of saturated fatty acids and monounsaturated fatty acids with the double bond at the uneven position, an additional enzyme is required for mono- and polyunsaturated fatty acids with the double bond at the even position to prepare them for β-oxidation: NADPH-dependent 2,4-dienoyl-CoA reductase (Figure 1). This enzyme was found in mammalian mitochondria and in peroxisomes of fungi (Kunau *et al.* 1988).

We recently demonstrated that peroxisomes *in vivo* are impermeable to NAD(H) and other small molecules and we were intrigued by the NADPH dependence of 2,4-dienoyl-CoA reductase activity. How is NADPH required for polyunsaturated fatty acid oxidation regenerated in these peroxisomes? To avoid the staggering complexity of isoenzymes involved in fatty acid β-oxidation in mammalian cells, we selected *S.cerevisiae* as a simple eukaryote model to analyse this question in further detail. Here we had to deal with only a single compartment, the peroxisome, in which β-oxidation takes place and the existence of yeast peroxisomal isoenzymes is thus far undocumented.

We extended the observations of Dommes *et al.* (1983) and confirmed the presence of NADPH-dependent 2,4-dienoyl-CoA reductase in peroxisomes of *S.cerevisiae* using subcellular fractionation experiments. A search with the amino acid sequence of 2,4-dienoyl-CoA reductase of *E.coli* in the yeast protein database revealed a homologous reading frame YNL202w, a sporulation-specific protein with similarity to human mitochondrial 2,4-dienoyl-CoA reductase. Furthermore, the predicted amino acid sequence revealed the presence of a C-terminal peroxisomal targeting signal, SKL (PTS1). The 5′ preceding DNA sequence contained an upstream activation sequence (UAS) with similarity to OREs, found in many genes coding for peroxisomal matrix proteins (Einerhand *et al.* 1993). In addition, we found that this enzyme contains a typical PTS1 matrix import signal that is dependent on the PTS1 receptor (Pex5p) for its import into peroxisomes and is induced on oleate (Kal, 1997). Growth on a polyunsaturated fatty acid like docosahexaenoic acid requires stoichiometric amounts of NADPH for its preparation for β-oxidation (Figure 1). NADPH for reductive processes is generated in the cytosol, in for instance the pentose phosphate pathway, and, since a direct transfer of reducing equivalents from NADH to NADP, as can take place via the transhydrogenase reaction in mitochondria (Rydstrom *et al.*, 1971), is not known for peroxisomes, we considered it likely that cytosolic NADPH is the primary source of reducing power for intraperoxisomal NADPH-dependent 2,4-dienoyl-CoA reductase. The impermeability of the peroxisomal membrane towards pyrimidine nucleotides implies the existence of a transport shuttle similar to the glycerol-3-phosphate and malate–aspartate shuttles responsible for transfer of reducing equivalents across the mitochondrial inner membrane (Elgersma and Tabak, 1996).

Recently, NADP-dependent isocitrate dehydrogenase was found in peroxisomes of the *n*-alkane-utilizing yeast *C.tropicalis* (Yamamoto *et al.*, 1995). This attracted our attention to the possible existence of an NADP-dependent isocitrate dehydrogenase shuttle to regenerate intraperoxisomal NADPH consumed during oxidation of polyunsaturated fatty acids. Indeed, when we searched the *S.cerevisiae* genome database, we found a gene encoding a third NADP-dependent isocitrate dehydrogenase isoenzyme in addition to the *IDP1* and *IDP2* genes coding for mitochondrial and cytoplasmic NADPH-dependent isocitrate dehydrogenase, respectively. The *IDP3* gene is preceded by an ORE, a UAS observed in many genes coding for peroxisomal matrix enzymes, and is highly expressed in a Pip2p transcription factor-dependent manner on oleate. The C-terminal part of the encoded protein is longer than that of the other isoenzymes, and the last three amino acids comprise a putative PTS1 according to the PTS1 consensus motif derived for *S.cerevisiae* (Elgersma *et al.*, 1996). Using cell fractionation studies and immunoelectron microscopy, we showed that Idp3p is a peroxisomal matrix enzyme that is dependent on the PTS1 receptor (Pex5p) for its import into peroxisomes. Disruption of the *IDP3* gene was associated with an almost complete block in growth on media containing arachidonic, linoleic and petroselinic acid as sole carbon source, due to the deficient oxidation of these fatty acids. Growth on oleate and oleate oxidation in cell-free extracts, however, was normal in Δ*idp3* cells, suggesting that the block in oxidation of arachidonic, linoleic and petroselinic acid is related directly to the position of the double bond at the even position in these fatty acids and not to the oxidation process *per se*. Indeed in cell lysates, in which

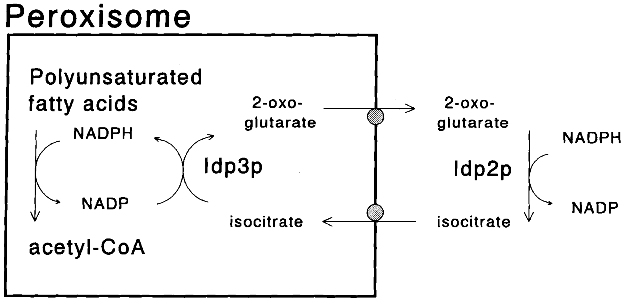

Fig. 12. Model for reduction of intraperoxisomal NADP. Our results do not rule out that metabolites other than 2-oxoglutarate or isocitrate are shuttled between peroxisome and cytosol.

the membrane barriers were disrupted by detergent and enzymatic reduction of NADPH is not required since NADPH is one of the components of the reaction medium, oxidation of docosahexaenoic acid was found to proceed normally. In intact cells, however, β-oxidation of docosahexaenoic acid was deficient.

These results support the existence of an isocitrate–2-oxoglutarate redox shuttle (Figure 12) to interconnect the cytosolic and peroxisomal pools of NADPH, and provide additional evidence for the impermeability of the peroxisomal membrane towards small molecules (Van Roermund *et al.*, 1995). Interestingly, a similar isocitrate–2-oxoglutarate redox shuttle was shown to exist linking the mitochondrial and cytoplasmic pools of NADPH in rat liver (Papa, 1969; Hoek *et al.*, 1974). Thus, all three NADP-dependent isocitrate dehydrogenases are involved in a similar process of shuttling reducing equivalents between NADPs of different subcellular compartments.

Materials and methods

Yeast strains and culture conditions

The wild-type yeast strain used in this study was *S.cerevisiae* BJ 1991*(MATα, leu2, trp1, ura3-52, prb1-1122, pep4-3, gal2)*. The Δ*pex5*, Δ*pex6* and Δ*pex7* mutants used were isolated by Van der Leij *et al.* (1992) and Voorn-Brouwer *et al.* (1993). Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO) (Difco), 0.3% glucose or 2% glucose and amino acids (20–30 μg/ml) as required. The liquid media used for growing cells for nucleic acid isolation, growth curves, subcellular fractionation, β-oxidation assays, immunogold electron microscopy and enzyme assays contained 0.5% potassium phosphate buffer pH 6.0, 0.3% yeast extract, 0.5% peptone, and either 2% glucose, 3% glycerol, 2% K-acetate, 0.1% oleate/0.2% Tween-40, 0.1% petroselinic/0.2% Tween-40 or 0.1% linolenic acid/0.2% Tween-40 with amino acids as needed. Before shifting to these media, the cells were grown on minimal 0.3% glucose medium for at least 24 h.

Oleic, linoleic, arachidonic and petroselinic acid plates contained 0.67% yeast nitrogen base without amino acids (YNB-WO) (Difco), 0.1% yeast extracts, 2% agar, amino acids as needed, and 0.1% fatty acid/0.25% Tween-40.

Cloning procedures

Standard DNA techniques were carried out as described by Sambrook *et al.* (1989). The yeast *IDP3* gene was amplified using two primers corresponding to specific regions of non-homology with *IDP1* or *IDP2*, regions outside both sides of the open reading frame (ORF). The yeast *IDP3* gene was amplified from genomic DNA using the 5′ IDP3 (–633) primer (5′-GTGCTGCAAAAGAATGTG-3′) and the 3′ *IDP3* (2012) (+*Bam*HI site) primer (5′-TTTGGATCCAGAGTGATCTCAGAAGCC-3′). The resulting 2.6 kb fragment was digested with *Eco*RI and *Bam*HI and was subcloned in pUC19. The whole ORF was deleted by replacing the *Pst*I–*Xba*I fragment (containing 1263 bp of the *IDP3* ORF) by the *LEU2* gene. Leu$^+$ transformants were selected for integration in the *IDP3* gene by PCR analysis.

For all expression constructs described, the single-copy or multicopy catalase A (*CTA1)* promoter expression plasmids based upon YcPlac33 and YcPlac181 were used, as described by Gietz and Sugino (1988) and Elgersma *et al.* (1995). For the in-frame fusion of the *IDP2* and *IDP3* genes with the NH epitope, a *Bam*HI restriction site was introduced in front of both ORFs. PCR was performed under standard conditions using the *Bam*HI *IDP2* primer (5′-TTTGGATCCATGACAAAGATTAA-GGTA-3′) and the *IDP2* (1884) primer for the *IDP2* gene, and a PCR was performed under normal conditions using the *Bam*HI *IDP3* primer (5′-TTTGGATCCATGAGTAAAATTAAAGTTGTTC-3′) and the 3′*IDP3* (2012) primer. The *Bam*HI–*Pst*I *IDP2* and the *Bam*HI–*Xba*I *IDP3* PCR fragments were introduced in a single and multicopy *CTA1* expression plasmid containing the NH tag, resulting in an ORF encoding NH-tagged Idp2p and the NH-tagged Idp3p. All single and multicopy plasmids described contained the *URA3* gene as an auxotrophic marker.

The *IDP2* promotor was amplified by PCR using genomic DNA from BJ1991 with primers (–597) (5′-GGATCAACTCTTCATCGC-3′) and *IDP2* (+19) (5′-ATTTCCACCATGGGGTTAGC-3′). The PCR fragment was digested with *Hin*dIII and *Nco*I. The luciferase ORF was isolated from pDR101 with *Nco*I and *Bam*HI. Both fragments were ligated simultaneously into *Hin*dIII–*Bam*HI-digested YCPlac33, resulting in pAK87. The *IDP3* promotor was amplified by PCR using genomic DNA from BJ1991 with primers *IDP3* (–633) (5′-GTGCTGCAAAA-GAATGTG-3′) and *IDP3* (+19) (5′-CCATTTCCACCATGGGATG-AAC-3′). The PCR fragment was digested with *Eco*RI and *Nco*I. The luciferase ORF was isolated from pDR101 with *Nco*I and *Bam*HI. Both fragments were ligated simultaneously into *Eco*RI–*Bam*HI-digested YCPlac33, resulting in pAK88.

Subcellular fractionation and Nycodenz gradients

Subcellular fractionations were performed as described by Van der Leij *et al.* (1992). Organellar pellets were used for continuous 15–35% Nycodenz gradients (10 ml), with a cushion of 1.5 ml of 50% 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. at 4°C.

Preparation of extracts and TCA lysates

Cells were harvested, washed twice in water and extracts were prepared by breaking with glass beads in a buffer containing 200 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 10% glycerol (v/v). Cell debris was removed by centrifigation for 30 min at 13 000 r.p.m. in an Eppendorf centrifuge.

Of the fractions from the subcellular fractionation or Nycodenz gradient, 100 μl was collected in a 2 ml Eppendorf tube together with 900 μl of 11% trichloroacetic acid (TCA). After being left overnight, samples were centrifuged for 15 min at 12 000 r.p.m. at 4°C. The pellet obtained was resuspended in Laemmli sample buffer for SDS–PAGE analysis.

Western blotting

Proteins were separated on 12% SDS–polyacrylamide gels and transferred to a nitrocellulose filter in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The blots were blocked by incubation in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). The same buffer was used for incubation with the primary antibodies and with IgG-coupled alkaline phophatase. The blots were stained in AP buffer [100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 5 mM $MgCl_2$] with BCIP and NBT following the manufacturer's instructions (Boehringer Mannheim).

Electron microscopy

Oleate-induced cells were fixed with 2% paraformaldehyde and 0.5% glutaraldehyde. Ultra-thin sections were prepared as described by Gould *et al.* (1990).

NH epitope tagging and antibodies

The synthetic NH epitope tag CQDLPGNDNST (corresponding to the NH_2-terminus of the mature haemagglutinin protein) was conjugated to keyhole limpet haemocyanin by means of maleimide bis *N*-hydroxysuccinimide and used for antibody production in rabbits. For epitope tagging, an oligonucleotide adaptor encoding the NH epitope was ligated in the *Sac*I–*Bam*HI site of the *CTA1* expression plasmids (Elgersma *et al.*, 1996).

β-oxidation measurements

β-oxidation assays in intact cells were done essentially as described by Van Roermund *et al.* (1995) with the following modifications. Incubations were performed at 28°C and substrates were solubilized in 1 mg/ml α-cyclodextrine, 10 mM Tris (pH 8.0). The substrates used were [1-^{14}C]oleic acid and [1-^{14}C]docosahexaenoic acid.

Fatty acid β-oxidation activities were also measured in cell-free lysates prepared by lysing protoplasts or in an organellar pellet fraction in an assay medium containing the following components: 150 mM Tris (pH 8.5), 10 mM ATP, 10 mM $MgCl_2$, 50 μM FAD, 0.5 mM NAD, 0.5 mM NADPH, 0.5 mM CoA, 0.5 U/ml acyl-CoA synthetase (Boehringer Mannheim) and 10 μM [1-^{14}C]docosahexaenoic acid. Reactions were followed over time.

Identification of 2,4-dienoyl-CoA intermediates

In order to identify which acyl-CoA ester is accumulating in Δ*idp3* mutant cells, oleate-induced cells were incubated with 2.5 μM [1-^{14}C]docosahexaenoic acid as described above, for 30 and 60 min. Reactions were terminated by the addition of 50 μl of 2.6 M perchloric acid. In order to hydrolyse all CoA esters, 100 μl of 2 M NaOH was added to the mixture and incubations were allowed to proceed for at least 15 min at 50°C. This was followed by addition of ~150 μl of 0.5 M H_2SO_4, and 75 μl of sodium acetate buffer. If required, the pH was adjusted to 4.0. Fatty acids were then extracted with methanol/chloroform/heptane as described by Van Roermund *et al.* (1995). The lower layer was collected, and dried under nitrogen. The residue was taken up in acetone, and analysed by thin-layer chromatography as described by Bremer and Wojtczak (1972), with the exception that benzene was substituted for toluene. After 1 h, the plate was dried and exposed for 4 days on a phosphorimager.

The standard radioactively labelled [1-^{14}C](2,4,7,10,13,16,19) docosaheptaenoic acid (C22:7) was synthesized as described below.

Enzyme assays

3-hydroxyacyl-CoA dehydrogenase activities were measured on a Cobas-Fara centrifugal analyser by following the acetoacetyl-CoA-dependent rate of NADH consumption at 340 nm (Wanders *et al.*, 1990). NADP-dependent isocitrate dehydrogenase was measured on a Cobas-Fara centrifugal analyser by the method described by Loftus *et al.* (1994). Succinate dehydrogenase was measured according to the method of Munujos *et al.* (1993). 2,4-dienoyl-CoA reductase was measured by the method described by Nada *et al.* (1992). The substrate (2,4,7,10,13,16,19) docosaheptaenoyl-CoA was synthesized enzymatically from docosahexaenoic acid using acylCoA-synthetase (Boehringer) to synthesize the CoA-ester and acyl-CoA oxidase to generate the *trans* 2,3-double bond. Incubation conditions were as follows: 10 mM ATP, 10 mM $MgCl_2$ and 0.5 mM CoA and 0.2 M phosphate buffer pH 8.0. Luciferase activity was measured as described by Einerhand *et al.* (1993). Catalase A activity was measured as described by Lucke *et al.* (1963). Protein concentration was determined by the bicinchoninic acid method (Smith, 1985).

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