Molecular characterization of carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria in *Saccharomyces cerevisiae* and identification of a plasma membrane carnitine transporter, Agp2p

Carlo W.T.van Roermund¹, Ewald H.Hettema², Marlene van den Berg², Henk F.Tabak² and Ronald J.A.Wanders¹,³,⁴

University of Amsterdam, Academic Medical Centre, Departments of ¹Clinical Chemistry, ²Biochemistry and ³Paediatrics, Emma Children’s Hospital, PO Box 22700, 1100 DE, Amsterdam, The Netherlands

⁴Corresponding author e-mail: wanders@amc.uva.nl

**Introduction**

The β-oxidation of fatty acids in mammalian cells takes place in both mitochondria and peroxisomes. Long-chain fatty acids are oxidized primarily in mitochondria whereas very-long-chain fatty acids and certain branched-chain fatty acids are handled primarily by peroxisomes (Schulz, 1991; Seedorf et al., 1994; Dieuaidu-Noubhani et al., 1996; Leenders et al., 1996; Jiang et al., 1997; Wanders et al., 1997). The importance of peroxisomal fatty acid β-oxidation is emphasized by the existence of inherited diseases in man (e.g. X-linked adrenoleukodystrophy) that are caused by an impairment in peroxisomal β-oxidation (Wanders et al., 1995).

It is generally accepted that mammalian fatty acid β-oxidation in peroxisomes is incomplete and only involves chain shortening of fatty acids to produce acetyl-CoA and/ or propionyl-CoA plus medium-chain acyl-CoAs. These are then transported to the mitochondria as carnitine esters, where they are further oxidized to CO₂ and H₂O (Bieber, 1988; Osmundsen et al., 1991; Reddy and Manninaerts, 1994) as shown convincingly for pristanic acid (Verhoeven et al., 1998).

In contrast to mammals, degradation of fatty acids in yeast takes place exclusively in peroxisomes (Kunau et al., 1995). The acetyl-CoA produced has to be transported from the peroxisomes to the mitochondria for complete oxidation to CO₂ and H₂O. Two pathways for the transport of acetyl units have been identified (van Roermund et al., 1995). In the first, acetyl-CoA enters the peroxisosomal glyoxylate cycle to produce succinate, which is subsequently transported to the mitochondria, probably via the putative dicarboxylate carrier, Acr1p (Palmieri et al., 1997). The second pathway involves the intraperoxisomal conversion of acetyl-CoA into acetyl-carnitine, which is catalysed by carnitine acetyltransferase (Cat2p). The peroxisomal and mitochondrial Cat2p of *Saccharomyces cerevisiae* are encoded by a single gene *CAT2* (Elgersma et al., 1995) and are responsible for >95% of the total carnitine acetyltransferase activity in oleate-grown yeast cells (Kispal et al., 1993). The existence of two pathways for the transport of acetyl units from peroxisomes to mitochondria, which are acting in parallel, became clear with the finding that disruption of either the *CIT2* gene, encoding the peroxisomal glyoxylate cycle enzyme citrate synthase (Cit2p), or the *CAT2* gene did not affect growth of yeast on oleate, whereas a mutant with both genes disrupted (*Δcit2/cat2*) failed to grow on oleate, due to the inability to oxidize this fatty acid (van Roermund et al., 1995).

Based on these findings, we developed a selective screen for the isolation of mutants that are specifically defective in the carnitine-dependent acetyl-unit transport from peroxisomes to mitochondria (CDAT mutants). In this paper we report the isolation and characterization of various mutants, which could be assigned to three distinct complementation groups. The gene mutated in the first complementation group is the *CAT2* gene, which codes for both the peroxisomal and mitochondrial Cat2p proteins. The genes affected in the remaining two complementation groups were identified by functional complementation of the mutants. In complementation group 2, a previously uncharacterized gene was mutated, known as *YOR100c*. This gene encodes a member of the mitochondrial carrier family, which appears to be the orthologue of the human mitochondrial carnitine acylcarnitine translocase. In complementation group 3 the *AGP2* gene was mutated.
Although previously reported to be a general amino acid permease, we provide evidence that Agp2p is required for the uptake of L-carnitine from the medium into the yeast cell.

**Results**

**Isolation of CDAT mutants defective in the carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria**

Oxidation of straight-chain fatty acids in yeast is confined to peroxisomes and generates acetyl-CoA as the end product. Our previous studies have shown that transport of acetyl-CoA from peroxisomes to mitochondria may proceed via two independent pathways (Figure 1) namely, the glyoxylate cycle (pathway 1) or the carnitine-dependent pathway (pathway 2). Fatty acid oxidation and growth on oleate, which requires β-oxidation, were completely normal if either of the two pathways were blocked. However, mutants in which both pathways were blocked showed deficient fatty acid oxidation and impaired growth on oleate, indicating that the two pathways function in parallel.

In order to identify the components required for the carnitine-dependent acetyl-CoA transport from peroxisomes to mitochondria, we first deleted the gene coding for Cit2p, the peroxisomal citrate synthase, thereby blocking the glyoxylate cycle (pathway 1) or the carnitine-dependent pathway (pathway 2). Fatty acid oxidation and growth on oleate, which requires β-oxidation, were completely normal if either of the two pathways were blocked. However, mutants in which both pathways were blocked showed deficient fatty acid oxidation and impaired growth on oleate, indicating that the two pathways function in parallel.

In order to identify the components required for the carnitine-dependent acetyl-CoA transport from peroxisomes to mitochondria, we first deleted the gene coding for Cit2p, the peroxisomal citrate synthase, thereby blocking the glyoxylate cycle (pathway 1) or the carnitine-dependent pathway (pathway 2). Fatty acid oxidation and growth on oleate (Figure 1), was subsequently mutagenized by ethyl methyl sulfonate (EMS) treatment followed by the isolation of mutants that no longer grew on oleate (see Materials and methods). In principle, these mutants could be affected either in peroxisome biogenesis (pex mutants; Distel et al., 1996) or fatty acid β-oxidation.

In order to distinguish between these two possibilities, a construct expressing green fluorescent protein (GFP) with the C-terminal peroxisomal targeting signal PTS1 was transformed into the Δcit2 cells prior to mutagenesis.

Of the 99 mutants deficient for oleate growth, 21 appeared to have GFP–PTS1 mislocalized to the cytoplasm. Since this is the expected phenotype for pex mutants, these mutants were excluded from further analysis.

To distinguish between mutants affected in β-oxidation and in carnitine-dependent transport, we transformed the remaining 78 mutants with the CIT2 gene. Ten mutants regained the ability to grow on oleate plates, which made them candidates for mutants with a specific defect in the carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria (Table I).

<table>
<thead>
<tr>
<th>CDAT mutants</th>
<th>CIT2</th>
<th>CAT2</th>
<th>YOR100C</th>
<th>AGP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDAT-1</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>CDAT-2</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CDAT-3</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>CDAT-4</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CDAT-5</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>CDAT-6</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>CDAT-7</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>CDAT-8</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CDAT-9</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Δcit2/cit2</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Δcit2/cac</td>
<td>++</td>
<td>–</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
<td>Δcit2/agp2</td>
<td>++</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>Wild-type</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

CDAT mutants transformed with the genes coding for citrate synthase (CIT2), carnitine acetyltransferase (CAT2), carnitine acylcarnitine translocase (YOR100C) or the amino acid permease (AGP2) were able (+) or unable (–) to grow on oleate-containing plates. As positive and negative controls wild-type cells and Δcit2/cit2, Δcit2/cac and Δcit2/agp2 deletion mutants were used.

Characterization of the CDAT mutants

Carnitine acetyltransferase (CAT2). Earlier studies have established that Cat2p, which is located both in peroxi-
somes and mitochondria (Elgersma et al., 1995), is involved in the transport of acetyl-CoA from the peroxi-
somal matrix to mitochondria. It was therefore anticipated
that some of the CDAT mutants would be mutated in
the CAT2 gene. In order to identify these mutants, we
transformed the 10 CDAT mutants with the wild-type
CAT2 gene (Table I) and found restoration of growth on
oleate in four mutant strains (CDAT-2, -4, -8 and -10).

Cloning of YOR100C. The genes affected in the remaining
six mutants were identified by functional complementation,
i.e. restoration of growth on oleate following transforma-
tion with a genomic DNA library. Four mutant strains
(CDAT-1, -5, -7 and -9) appeared to be affected in
YOR100C, which has previously been reported to code
for an oleate-inducible mitochondrial protein (Karpichev
and Small, 1998). This gene was identified in the yeast
gene sequencing project and predicted to encode a
and Small, 1998). This gene was identified in the yeast
genome sequencing project and predicted to encode a
and Small, 1998). This gene was identified in the yeast
gene sequencing project and predicted to encode a

Localization and characterization of Yor100cp
To study the subcellular localization of Yor100cp in
oleate-grown cells, we epitope-tagged the protein at its
N-terminus with the NH-tag (see Materials and methods).
This construct was able to complement the Δcit2/yor100c
mutant indicating that the NH-tag does not interfere with
the protein’s function. After subcellular fractionation of
the oleate-grown transformant, we found NH-Yor100cp
to be present exclusively in the organellar pellet
(Figure 2A). To separate the peroxisomes from the mito-
ochondria, the organellar pellet was further fractionated by
Nycodenz density gradient centrifugation. Immunoblot
analysis of the gradient fractions using the NH-antibodies
revealed that NH-Yor100cp co-localized with the mito-
ochondrial marker indicating a localization in mitochondria
(Figure 2B). This was confirmed by immunoelectron
microscopy of oleate-induced cells expressing the NH-
Yor100cp from a single copy plasmid. Figure 2C and D
shows exclusive labelling of the mitochondrial inner
membrane (cristae). This result indicates that the product
of the YOR100C gene is a mitochondrial inner mem-
brane protein.

In order to investigate the function of Yor100cp, we
disrupted the YOR100C gene in wild-type cells and in the
Δcit2 mutant, by replacing YOR100C with the KAN gene
(see Materials and methods). The resulting deletion mutant
grew normally on glucose and glycerol. In contrast to the
deletion mutants created from the wild-type strain, which
grew normally, growth of Δcit2/yor100c cells on oleate
was strongly impaired. To establish whether Yor100cp functions as the yeast carnitine acylcarnitine translocase as predicted from its similarity to the human protein, we studied the oxidation of [1-14C]oleate in wild-type cells and deletion mutants. As shown in Figure 3, oxidation of [1-14C]oleate was strongly impaired only in the Δcit2–yor100c mutant but was normal in the other mutant strains. The ability to β-oxidize fatty acids could be restored by transformation with either the YOR100C or the CIT2 gene, indicating that the β-oxidation defect of Δcit2–yor100c cells on oleate is reversible.

If the block in β-oxidation of oleate in Δcit2–yor100c mutants is indeed caused by a defect in the transport of acetyl-CoA, or better still acetylcarnitine (Figure 1), from the peroxisomes to mitochondria as a result of the absence of the gene products of YOR100C and CIT2, this would be reflected in a defective import of acetylcarnitine into the mitochondria. This was tested by incubation of spheroplasts prepared from oleate-grown wild-type, Δyor100c, Δcit2 and Δcit2–yor100c cells for 10 min with [1-14C]acetylcarnitine in the presence of low concentrations of digitonin. As shown in Figure 4A, the oxidation of [1-14C]acetylcarnitine to [1-14C]CO2 was strongly impaired only in the Δcit2–yor100c, Δyor100c and Δcit2–yor100c mutants, whereas oxidation was normal in wild-type and Δcit2 cells. Oxidation was again normal in Δcit2–yor100c or Δyor100c cells that were transformed with the YOR100C gene.

Figure 4B shows the carnitine acylcarnitine translocase activities measured in wild-type cells grown on glucose, glycerol and oleate. The results show that the activity was repressed by glucose, derepressed by glycerol and induced by oleate, which illustrates that expression of YOR100C is similar to that of the β-oxidation enzymes such as 3-hydroxyacyl-CoA dehydrogenase (3HAD), as already predicted from the presence of the ORE box in the YOR100C promoter.

Taken together, our experiments show that the gene product of YOR100C is a member of the mitochondrial carrier family, which is induced on oleate, involved in the transport of acetyl-CoA from peroxisomes to mitochondria and functions as a carnitine acylcarnitine translocase in the mitochondrial inner membrane of *S. cerevisiae.*

**Cloning of the AGP2 gene, encoding a member of the amino acid permease family**

Functional complementation of the remaining two mutant strains (*CDAT-3* and *CDAT-6*) identified the YBR132C ORF as the affected gene (Table I). YBR132C is identical to the previously reported *AGP2* (André, 1995), a gene that codes for a 596 amino acid protein with 12 potential transmembrane domains and which belongs to the family of amino acid permeases. Proteins belonging to this family are assumed to function as plasma membrane proton-symporters. Inspection of the *S*-region of the *AGP2* gene revealed the presence of a putative ORE (Karpichev and Small, 1998).

**Localization and characterization of Agp2p**

To verify that Agp2p is required for oleate growth, we made a gene deletion of *AGP2* in wild-type and Δcit2 cells. Growth of the resulting deletion mutants was unaffected on rich glucose, glycerol or oleate media, except for the Δcit2/agp2 double mutant, which showed strongly impaired growth.
impaired growth on oleate, the same phenotype as observed for the original mutants.

To study the subcellular localization of the AGP2 gene product, Agp2p, we introduced the HA-tag at the C-terminus of the protein. This did not affect the function of the protein as demonstrated by the fact that this construct functionally complemented the Δcit2/agp2 double mutant. Using subcellular fractionation experiments, we studied the localization of Agp2p-HA in cells grown overnight in rich oleate medium. A total cellular extract was compared with homogenates prepared from spheroplasts by gentle osmotic lysis and fractionated by successive differential centrifugation steps into a 2500 g pellet (P1), a 17 000 g pellet (P2), a 100 000 g pellet (P3) and a supernatant fraction (S). All fractions were analysed for the presence of marker enzymes for various subcellular compartments, including mitochondria, peroxisomes, endoplasmic reticulum (ER) and plasma membrane. Agp2p-HA co-localized with all fractions and thus behaved differently to one of the marker enzymes (data not shown), suggesting that the protein has different locations inside the cell. This is in line with the observations of Ljungdahl et al. (1992), who indicated that Agp2p is localized in the plasma membrane and the ER.

To confirm the intracellular localization of Agp2p, we performed immunoelectron microscopy of oleate-induced cells expressing Agp2p-HA from a single copy plasmid. Figure 5 shows prominent labelling of the plasma membrane in addition to labelling of ER membranes and vacuoles in oleate-induced yeast cells.

**Agp2p functions as a carnitine transporter**

In order to investigate the role of Agp2p in the carnitine-dependent acetyl-CoA transport from peroxisomes to mitochondria, we measured the total intracellular carnitine levels in Δagg2 and Δcit2/agp2 cells (Figure 6) and found a profound decrease. Based on the prediction that Agp2p functions as a transporter in the plasma membrane, this observation strongly suggested that Agp2p is required for the uptake of carnitine from the medium. This would imply that the wild-type S. cerevisiae strain used for this study is not capable of synthesizing L-carnitine. Indeed, when we tested the Δcit2 strain on minimal oleate medium, we only observed growth after the addition of 20 μM L-carnitine (Figure 7B) but not in the absence of carnitine (Figure 7A), indicating that this strain is not capable of de novo carnitine synthesis. Growing these cells in the presence of high concentrations of L-carnitine (Figure 7C) could compensate for the defect in oleate growth introduced by the deletion of AGP2 in Δcit2 cells. In agreement with these results, incubating the cells with high concentrations of L-carnitine (Figure 7D) could also restore the impaired oxidation of oleate in Δcit2/agp2 cells.

To provide direct evidence for the function of Agp2p as a carnitine transporter, we measured the uptake of L-[1-14C]carnitine in wild-type and mutant cells grown on oleate-containing medium (see Materials and methods).
Fig. 7. (A–C) Growth of wild-type cells and mutant cells on minimal oleate medium containing no (A), 20 μM (B) or 500 μM L-carnitine (C). The following symbols were used: (■) wild-type cells, (+) Δaggp2 cells, (▲) Δcit2 cells, (●) Δcit2/aggp2. (D) Oleate β-oxidation is reduced in Δcit2/aggp2 cells. Wild-type and mutant cells grown on oleate were incubated with [1-14C] oleate without carnitine or with 20 μM or 500 μM carnitine and β-oxidation were measured as described in Materials and methods. The β-oxidation rates in wild-type cells were taken as reference (100%) and are expressed as the sum of [1-14C] CO2 and water-soluble β-oxidation products produced.

NEM is practically membrane impermeable, which confirms that Agp2p is functionally active in the plasma membrane of *S. cerevisiae*.

The uptake of L-[1-14C]carnitine was particularly high in cells at pH 5.0 (Figure 8B), which suggests that Agp2p functions as a carnitine–H+/H11001 symporter. Carnitine uptake was not sodium dependent since replacement of sodium with potassium did not affect the uptake (Figure 8C).

The concentration dependence of L-carnitine transport was examined to estimate the half-saturation concentration ($K_m$) of Agp2p-mediated L-carnitine transport. The half-saturation concentration and the maximum transport activity ($V_{	ext{max}}$) were estimated to be 5 μM and 21.2 nmol/mg/10 min, respectively (Figure 8D).

The specificity of Agp2p-mediated L-carnitine transport was examined in terms of the inhibitory effect on the initial uptake of L-[1-14C]carnitine. As is evident from Figure 8C, structurally analogous compounds including D-carnitine and acetylcarnitine reduced L-carnitine uptake at 10 μM. Accordingly, the structural requirement of Agp2p-mediated L-carnitine transport is rather strict. Amino acids including lysine, serine, leucine and threonine did not affect the uptake of L-carnitine.

Figure 8E shows the uptake of L-carnitine in wild-type cells grown on glucose, glycerol and oleate. The results show that L-carnitine uptake was repressed by glucose, derepressed by glycerol and induced by oleate, which indicates that the expression of Agp2p is similar to that of β-oxidation enzymes. Although this was also predicted from the presence of the ORE box in the *AGP2* promoter, it cannot be excluded at this point that the activity of Agp2p is also regulated at the protein level. Taken together, these data indicate that L-carnitine uptake is functionally linked to fatty acid oxidation.

**Discussion**

Peroxisomes are the exclusive site of fatty acid β-oxidation in *S. cerevisiae*. Since the peroxisomal membrane is impermeable to small molecules, the question arises as to how the acetyl-CoA produced in peroxisomes is transported to the mitochondria for final oxidation to CO2 and H2O. Our earlier studies (Elgersma et al., 1995; van Roermund et al., 1995) have shown that there are only two ways in which acetyl-CoA can leave the peroxisome (Figure 1). It was the purpose of this study to resolve the structure of pathway 2, which catalyses the carnitine-dependent transport of acetyl-CoA from peroxisomes to mitochondria. To this end, a negative selection screen was developed based on mutagenesis of the Δcit2 mutant in which pathway 1 is blocked (Figure 1). The 10 CDAT mutants identified in this screen were found to represent three different genes. The first gene was *CAT2*, which codes for both the peroxisomal and mitochondrial carnitine acetyltransferase activity in oleate-grown cells (Elgersma et al., 1995).

In addition to the four *cat2* mutants, four mutants were affected in the *YOR100C* gene, which we demonstrated...
Fig. 8. Carnitine uptake is reduced in Δagp2 cells. (A) Wild-type and mutant cells grown on oleate were incubated with L-[1-14C]-carnitine and L-carnitine uptake was measured (see Materials and methods). The uptake rates in wild-type cells were taken as reference (100%). The following symbols were used: (■) wild-type cells, (▲) Δagp2 cells, (+) Δagp2.pAgp2 cells, (○) wild-type.pAgp2 cells and (△) Δagp2.pAgp2 cells, (□) wild-type cells incubated with 10 μM NEM. (B) pH optimum of L-carnitine uptake in oleate-induced wild-type cells. (C) D-carnitine and acetylcarnitine reduced the L-carnitine uptake in oleate-induced wild-type cells. Amino acids including lysine, serine, leucine and threonine did not affect the uptake. The uptake rates in wild-type cells were taken as reference (100%). Cells were incubated for 10 min with L-[1-14C]-carnitine. (D) Concentration dependence of L-carnitine transport in oleate-induced wild-type cells. (E) L-carnitine uptake is induced by oleate. Wild-type cells grown on glucose, glycerol and oleate were incubated with L-[1-14C]-carnitine for 10 min and L-carnitine uptake was measured. The uptake rates in wild-type cells were taken as reference (100%).

to code for the carnitine acylcarnitine translocase, a member of the mitochondrial carrier family. This carrier catalyses the exchange between acylcarnitine and free carnitine and is present in the mitochondrial inner membrane of many eukaryotic species. That the YOR100C gene indeed codes for the functional orthologue of the human CACT has recently been confirmed by the functional complementation of the Δcit2/yor100c mutant with the human CACT cDNA (unpublished results). Following the rules for yeast gene nomenclature, we therefore propose
to rename the YOR100C gene into CAC (carnitine acylcarnitine carrier) and the encoded protein Capc.

Although we also predicted the existence of a similar carrier involved in the export of acetylcarnitine in the peroxisomal membrane, so far we have not identified candidate mutants. Several possibilities can be put forward to explain this. First, mutants affected in a putative peroxisomal carnitine acylcarnitine translocase may not be able to grow on oleate after transformation with the CIT2 gene. Secondly, the peroxisomal membrane may be permeable to acetylcarnitine. Thirdly, the YOR100C (CAC) gene may code for both the mitochondrial and peroxisomal carnitine acylcarnitine translocase, analogous to the YOR100C or CIT2 be able to grow on oleate after transformation with the peroxisomal carnitine acylcarnitine translocase may not explain this. First, mutants affected in a putative candidate mutants. Several possibilities can be put forward to identify these mutants for other, unknown, reasons (e.g. functional redundancy of carriers capable of acetylcarnitine export).

The third gene identified in our screen is ORF YBR132C (AGP2), which codes for Agp2p, a protein of 596 amino acids. Agp2p contains 12 potential transmembrane domains and is related to Put4p, Alp1p, Lyp1p, Can1p and Gap1p, which are all members of the family of amino acid permeases. Based on sequence similarity, Nelissen et al. (1997) and André (1995) described Agp2p as one of the 18 members of this family with an unknown function. Members of this family are initially inserted into the ER membrane (Green et al., 1989; Green and Walter et al., 1992) and subsequently translocated to the plasma membrane via the yeast secretory pathway. Degradation of such carriers occurs after uptake in the vacuole. Immunogold electron microscopy studies confirmed that Agp2p is located primarily in the plasma membrane, but also in the ER and the vacuole, which is in agreement with the findings of Ljungdahl et al. (1992).

Furthermore, our results show that Agp2p is induced by growth on oleate, regulates the carnitine level inside the cells and is essential for oleate growth only when the peroxisomal glyoxylate cycle is inactive as a result of the disruption of CIT2 (Figure 1). This indicates that intracellular carnitine is required for the export of acetyl-CoA to mitochondria by allowing the intraperoxisomal conversion of acetyl-CoA to acetylcarnitine. Interestingly, blocking of the glyoxylate cycle activity in wild-type cells results in an oleate-growth-deficient phenotype only during growth on minimal oleate (without carnitine), whereas there is normal growth if L-carnitine (20 μM) is added (Figure 7B) or on rich oleate medium that already includes ~20 μM carnitine, suggesting that these cells are incapable of de novo synthesis of carnitine.

Primary carnitine deficiency in man is caused by a deficiency of the active transport of carnitine across the plasma membrane, which is catalysed by a Na+/carnitine transporter (Tein et al., 1996). Recently, Tamai et al. (1998) cloned the human organic cation transporter, OCTN2, which is a sodium-ion-dependent, high-affinity carnitine carrier, and demonstrated that mutations in its gene are responsible for primary carnitine deficiency. Most adult tissues, including skeletal muscle, kidney, placenta and heart, show high expression of OCTN2 and have been reported to take up carnitine via a sodium ion-dependent, carrier-mediated transport mechanism (Rebouche, 1977; Mølstad et al., 1978; Vary and Neely, 1982; Bremer, 1983; Steiger et al., 1995; Prasad et al., 1996; Tein et al., 1996). Tissues that have apparently low-affinity carnitine transporters, such as liver, brain and intestine, showed low expression of OCTN2 (Tamai et al., 1998). Surprisingly, the carnitine transporter we identified did not show any sequence similarity to the human OCTN2 or to any human proteins currently present in the databases. Furthermore, in contrast to OCTN2, the carnitine transport by Agp2p is Na+ independent.

In conclusion, we identified three genes that are involved in the carnitine-dependent acetyl-CoA transport from peroxisomes to mitochondria in S.cerevisiae: CIT2, YOR100C (CAC) and AGP2. The identification of these three proteins contributes to a better understanding of the communication between peroxisomes and mitochondria, and sheds new light on the physiological and biochemical functions of carnitine.

Materials and methods

Yeast strains and culture conditions

The wild-type strain used in this study was S.cerevisiae B1991 (α leu 2-3, trp1, ura3-251, prb1-1122, pep4-3, gal2). The Δsli1 and Δac2 mutants have been described previously (Voom-Brouwer et al., 1995; Elgersma et al., 1995). Yeast transformants were selected and grown on minimal medium containing 0.67% yeast nitrogen base without amino acids (YNB-WO; Difco), supplemented with 0.3% glucose and amino acids (20 μg/ml) as needed. Liquid rich media used to grow cells for DNA isolation, growth curves, subcellular fractionation, β-oxidation assays, immunogold electron microscopy and enzyme assays were composed of 0.5% potassium phosphate buffer pH 6.0, 0.3% yeast extract, 0.5% peptone and either 3% glycerol or 0.12% oleate/0.2% Tween-40. Before shifting to these media, the cells were grown on minimal 0.3% glucose medium for at least 24 h. Minimal oleate medium contains YNB-WO supplemented with all amino acids and 0.12% oleate/0.2% Tween-40.

Mutant selection

Δsli2 cells were transformed with a GFP-PTS1 expression construct (Hettema et al., 1998) and grown on 0.3% glucose medium. Aliquots containing approximately 5 × 10^5 cells were treated with 1.5–3% EMS as described by Lawrence (1991). The EMS treatment was stopped by adding 10% (v/v) sodium thioglycollate. Survival in independent experiments varied between 44 and 68%. After washing, the EMS-treated cells were allowed to recover by growing them for 4 h in 10 ml of minimal medium containing 2% glucose and the appropriate amino acids (20 μg/ml). Following mutagenesis, cells were grown on plates containing 2% glucose and subsequently replica-plated onto glycerol and oleate plates. Using this method 99 mutants were selected that were deficient in oleate growth but capable of glycerol growth.

Cloning, sequencing and disruption of the YOR100C and AGP2 genes

The impaired growth of CDAT-1 and CDAT-3 cells on oleate plates was used for cloning of the YOR100c and the AGP2 genes, respectively, by functional complementation with an S.cerevisiae genomic library. The transformants were selected on plates containing glucose and subsequently replica-plated onto glycerol or oleate plates. Different plasmids were selected for further characterization (pCDAT1.1, pCDAT1.2, pCDAT1.3 or pCDAT3.1, pCDAT3.2, pCDAT3.3). Complementing plasmids were rescued in Escherichia coli and retransformed to CDAT-1 and CDAT-3 cells to confirm linked complementation. The genomic insert of the complementing plasmids was sequenced by the dyeoxy-chain-termination method. The obtained nucleotide and predicted amino acid sequences were compared with the S.cerevisiae Genome Database, which led to the identification of YOR100C and YBR132C (AGP2) as the genes involved.

To construct ΔYOR100c or ΔAGP2 deletion mutants, the entire YOR100C or AGP2 ORF was replaced by the kanMX marker gene
and subsequently deproteinized with 500 μl of 0.1 M glycine, 20% methanol). The blots were blocked by incubation in 1% BSA in PBS, with 1% bovine serum albumin (BSA).

Preparation of extracts

Cells were harvested, washed twice in water and extracts were prepared in a buffer containing 200 mM Tris–HCl pH 8.0, 1 mM EDTA, 1 mM KCl and 8.5% glycerol (v/v) by disrupting the cells with glass beads on a vortex. The cell debris (75 μl) was mixed with 150 μl of each fraction from the Nycodenz gradient was used for precipitation in a 2 ml Eppendorf tube together with 1350 μl of 11% (w/v) trichloroacetic acid (TCA). After being left overnight at 4°C, samples were centrifuged for 15 min at 12 000 r.p.m. at 4°C. The pellet obtained was resuspended in 100 μl Laemmli sample buffer and used for SDS–PAGE analysis.

Western blotting

Proteins were separated on 12% SDS–polyacrylamide gels and transferred onto nitrocellulose filters in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol) followed by the manufacturer’s instructions (Boehringer Mannheim).

Electron microscopy

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

NH- and HA-epitope tagging and antibodies

For epitope tagging of proteins two different epitopes were used. The first was the NH-epitope, with a sequence QQLPQDNSTAGGSG, which corresponds to the N-terminus of the mature haemagglutinin protein and is recognized by a polyclonal antiserum. To introduce the NH-epitope tag at the C-terminus of proteins, an oligonucleotide adaptor encoding the NH-epitope was ligated to the cytosolic DnaJ-like protein and is recognized by a polyclonal antiserum. To introduce the NH-epitope tag at the C-terminus of proteins, an oligonucleotide adaptor encoding the NH-epitope was ligated into the Put1–HindIII site of the single-copy catalase A (CTA1) expression plasmid as described by Elgersma et al. (1996). The second tag was the HA-epitope with the sequence YDVPDYASLKE*, which is recognized by the monoclonal antibody 12CA5. To introduce the HA-epitope tag at the C-terminus of proteins, an oligonucleotide adaptor encoding the HA-epitope was ligated into the Put1–HindIII site of the single-copy catalase A (CTA1) expression plasmid as described by Elgersma et al. (1996).

Total carnitine measurements

Twenty millilitres of oleate-grown cells (OD = 1.5) were washed twice and disrupted by vigorously vortexing for 30 min at 4°C with ~200 μl glass beads in an end volume of 400 μl. Cell debris (75 μl) was mixed and subsequently deproteinized with 500 μl acetonitrile and centrifuged (12 000 r.p.m., 15 min). The resulting supernatant was dried under nitrogen at 45°C and subsequently derivatized in 100 μl butanol–HCl for 15 min at 60°C. Samples were then dried under nitrogen at 45°C and redissolved in 140 μl acetonitril. Free carnitine was measured as described by Vreken et al. (1999).

Enzyme assays

β-oxidation assays in intact cells were performed as described previously by Van Roermund et al. (1998). The carnitine acylcarnitine translocase activity was measured in spheroplasts prepared from wild-type or mutant cells grown on oleate. Activity measurements were performed in a medium containing 1.2 M sorbitol, 50 mM KCl, pH 7.5, 1 mM EDTA, 200 000 d.p.m. [1-14C]acetyl-carnitine (5 μM) and digitonin (20 μg/ml) spheroplasts (100 μg protein). This digitonin concentration selectively permeabilizes the plasma membrane, as demonstrated by complete release of the cytosolic marker enzyme phosphoglucone isomerase (PGI), whereas intracellular membranes of mitochondria and peroxisomes remain intact (Verleur et al., 1997). Reactions were allowed to proceed for 10 min at 28°C, and subsequently stopped by the addition of 100 μl 1.3 M perchloric acid. Radiolabelled CO2 was trapped overnight in 500 μl of 2 M NaOH.

3-hydroxyacyl-CoA dehydrogenase activity was measured on a Cobas-Fara centrifugal analyser by monitoring the acetoacetyl-CoA-dependent rate of NADH consumption at 340 nm (Wanders et al., 1992). Fumarase activity was measured on a Cobas-Fara centrifugal analyser by monitoring APADH production at 365 nm. The reaction was started with 10 mM fumarate in an incubation mixture of 100 mM Tris pH 9.0, 0.1% Triton-X-100, 4 U/ml malate dehydrogenase (Boehringer) and 1 mM APAD for 5 min at 37°C. Protein concentrations were determined by the bicinchoninic acid method described by Smith (1985).

Acknowledgements

We are grateful to Dr H.Waterham for critical reading of the manuscript and participation in the final stages of this work, to L.Hist for stimulating discussions, to Dr Y.Elgersma for providing the genomic DNA library to G.E.Mocht for technical assistance and to Dr P.van der Sluijs for the NH-epitope and NH-antiserum.

References


