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Review

Identification of mitochondrial carriers in *Saccharomyces cerevisiae* by transport assay of reconstituted recombinant proteins

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Abstract

The inner membranes of mitochondria contain a family of carrier proteins that are responsible for the transport in and out of the mitochondrial matrix of substrates, products, co-factors and biosynthetic precursors that are essential for the function and activities of the organelle. This family of proteins is characterized by containing three tandem homologous sequence repeats of approximately 100 amino acids, each folded into two transmembrane α-helices linked by an extensive polar loop. Each repeat contains a characteristic conserved sequence. These features have been used to determine the extent of the family in genome sequences. The genome of *Saccharomyces cerevisiae* contains 34 members of the family. The identity of five of them was known before the determination of the genome sequence, but the functions of the remaining family members were not. This review describes how the functions of 15 of these previously unknown transport proteins have been determined by a strategy that consists of expressing the genes in *Escherichia coli* or *Saccharomyces cerevisiae*, reconstituting the gene products into liposomes and establishing their functions by transport assay. Genetic and biochemical evidence as well as phylogenetic considerations have guided the choice of substrates that were tested in the transport assays. The physiological roles of these carriers have been verified by genetic experiments. Various pieces of evidence point to the functions of six additional members of the family, but these proposals await confirmation by transport assay. The sequences of many of the newly identified yeast carriers have been used to characterize orthologs in other species, and in man five diseases are presently known to be caused by defects in specific mitochondrial carrier genes. The roles of eight yeast mitochondrial carriers remain to be established.
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1. Introduction

The inner membranes of mitochondria contain a variety of proteins that transport metabolites, nucleotides and co-factors in and out of the matrix [1]. In early studies from 1974 to 1997, the primary structures of the mitochondrial ADP/ATP carrier [2], the uncoupling protein [3], the carriers for phosphate [4], oxoglutarate/malate [5], citrate [6], and carnitine/acylcarnitine [7] were elucidated using proteins purified from mitochondria. This work led to the important conclusion that these different transporters belong to the same protein family named "the mitochondrial carrier family". Thus, all of them have a tripartite structure consisting of three tandemly repeated sequences about 100 amino acids in length [8]. The repeats of one carrier are related to those found in the others. Each repeat contains 2 hydrophobic stretches that span the membrane as α-helices and a characteristic sequence motif P–X–D/E–X–X–K/R–X–K/R–(20–30 residues)–D/E–G–(5 residues)–K/R–G. This sequence motif is partially modified in one, two or even all three repeats in several mitochondrial carriers. Because of their presence in very

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minute amounts in the membrane the mitochondrial transporters were difficult to purify [9,10]. Consequently, the number of their sequences determined after purification was low.

In the post-genomic era, many more mitochondrial carriers have been identified in a relatively short time without the need for laborious purification of carriers from natural sources. In these studies, the characteristic sequence features of the mitochondrial carrier family were used to search the genomic sequences of various organisms for new members of the same family. The functions of many of these new family members were unknown.

When the genomic sequence of *Saccharomyces cerevisiae* was completed in 1996, we found that this genome encodes 35 mitochondrial carriers [11–13,but,see,14]. At the time, the functions of only five of these carriers were known, namely the three isoforms of the ADP/ATP carrier (Aac1p, Aac2p and Aac3p) [15–17] and the carriers for phosphate (Mir1p) [18] and citrate (Ctp1p) [19], which had been cloned on the basis of their relationship to the mammalian orthologs. To identify the functions of the remaining family members, we decided to express their genes in *Escherichia coli* and/or *S. cerevisiae*, incorporate the purified gene products into phospholipid vesicles (liposomes) and identify their functions by transport assays [see refs. 11,20–34 for description of the methods]. By means of this strategy, the functions of 15 new members of the mitochondrial carrier family in *S. cerevisiae* were identified. These new transporters are the dicarboxylate carrier (Dic1p) [11,22], the oxaloacetate carrier (Oac1p) [23], the two isoforms of the oxodicarboxylate carrier (Odc1p and Odc2p) [24], the carnitine carrier (Crc1p) [25], the aspartate/glutamate carrier (Agc1p) [26], the ornithine transporter (Ort1p) [27], the succinate-fumarate carrier (Sfc1p) [28], the *S*-adenosylmethionine transporter (Sam5p) [29], the thiamine pyrophosphate carrier (Tpc1p) [30], the peroxisomal adenine nucleotide transporter (Ant1p) [31], the pyrimidine nucleotide transporter (Pyt1p, known as Rim2p) [32], the GTP/GDP carrier (Ggc1p) [33] and the two isoforms of the NAD^+ transporter (Ndt1p and Ndt2p) [34]. This review focuses on the identification, the main transport properties and the physiological functions of these newly identified yeast transporters. It will also mention the transporters that have been identified in human and plant genomes based on the known yeast homologs.

2. Looking for new mitochondrial carriers in *S. cerevisiae*

The genome of *S. cerevisiae* was chosen as the focus of our efforts because it was the first to be sequenced completely in eukaryotes, it is manipulated easily, and there are extensive genetic data. Also, its metabolic pathways have been investigated thoroughly and are generally well known. Later, we extended our work to the human genome ([1] and references therein, 35–37) and to the genome of *Arabidopsis thaliana* ([38] and references therein, [39]).

Identifying the functions of members of the mitochondrial carrier family requires detection of the substrates transported by each carrier. We based our choices of potential substrates on the degree of sequence similarity of unknown carriers to known mammalian mitochondrial carriers, on genetic information and on knowledge of cell metabolism. In some cases, phylogenetic clustering of a particular carrier with others of known function provided a clue about which substrates were to be tested. Protein localization in mitochondria, or in one case in peroxisomes, also provided useful hints about carrier functions. Often, combinations of the above-mentioned criteria were used.

A phylogenetic tree of the *S. cerevisiae* mitochondrial transporters is presented in Fig. 1. This tree consists of 34 sequences since one of the previously considered mitochondrial carriers, i.e., the YDR470c gene product, named Ugo1p, is localized in the outer mitochondrial membrane, contains a single transmembrane domain, and is involved in the regulation of mitochondrial fusion [40].

2.1. Identification of carriers using sequence similarity

The "similarity approach" has been of limited value because the degree of similarity between the yeast carriers and the mammalian sequences of known function is generally not much higher than that between the different members of the mitochondrial carrier family. By screening the *S. cerevisiae* genome with the bovine sequence of the oxoglutarate/malate carrier (OGC) [5], five sequences were identified as Dic1p, Oac1p, Sfc1p, Odc1p and Odc2p (reported from the highest to lowest homology with OGC). Contrary to our expectations, the sequences closest to OGC (i.e., Dic1p, Oac1p and Sfc1p sharing 34%, 29% and 27% identical amino acids with OGC, respectively) transported substrates other than oxoglutarate. Moreover, Odc1p and Odc2p, which share a lower percentage (25%) of identical amino acids with OGC in comparison to the other carriers, transported oxoglutarate, although their substrate specificity differed from that of OGC. By screening the *S. cerevisiae* genome with the rat carnitine/acylcarnitine carrier [7] and the two isoforms of the human aspartate/glutamate carrier [41], Crc1p and Agc1p were found as the most closely related sequences, respectively. However, transport assays of the purified and reconstituted orthologs in yeast and mammals revealed that they differ markedly in several respects [25,26].

2.1.1. The dicarboxylate carrier Dic1p (YLR348c)

The closest yeast sequence to the bovine oxoglutarate carrier, namely the YLR348c gene product, was identified from its transport characteristics as the carrier for dicarboxylate ions [11,22]. The protein was isolated previously, but not sequenced, from the mitochondria of rat liver [42] and yeast [43]. Dic1p transports dicarboxylates, such as malate, succinate or malonate, and also inorganic phosphate by an obligatory counter-exchange mechanism. To a lesser extent, sulphate and thiosulphate are also exchanged by Dic1p for a dicarboxylate (e.g., malate) or phosphate. Furthermore, the yeast Dic1p is inhibited by the substrate analogues butylmalonate, benzylmalonate and phenylsuccinate. The transport affinity (Km) of Dic1p for phosphate (1.65 mM) is about 3-fold higher than that for malate (0.56 mM) [11]. All the transport properties of Dic1p closely resemble those determined previously for the dicarboxylate carrier in mitochondria [44,45] and after purification in reconstituted liposomes [42,46].

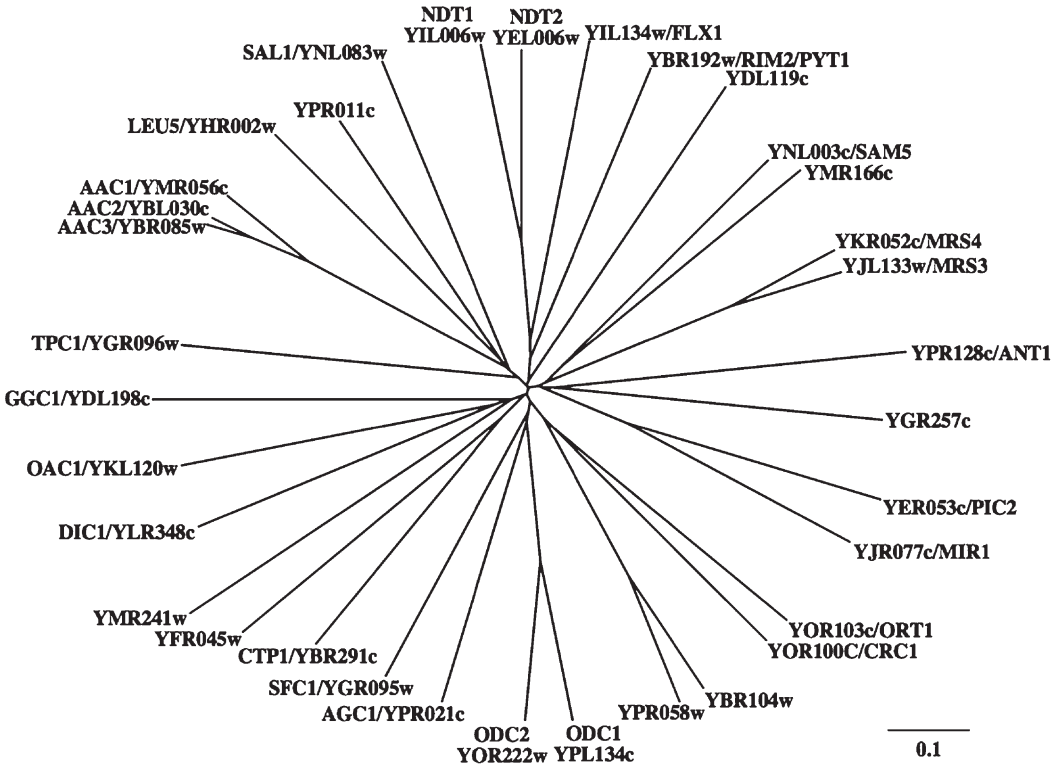

Fig. 1. Phylogenetic tree of the mitochondrial carrier family members of *Saccharomyces cerevisiae.* The tree originated from an alignment performed by CLUSTAL X (1.75) software using default parameters and visualized using Phylodendron TreePrint (http://www.es.embnet.org/Doc/phylodendron/treeprint-form.html). Branch lengths are drawn proportional to the amount of sequence change. The bar indicates the number of substitutions per residue with 0.1 corresponding to a distance of 10 substitutions per 100 residues. In the figure, the names of the genes corresponding to the ORF and those assigned by the *S. cerevisiae* Genome Database (SGD) are reported, except for NDT1 and NDT2 which are from Ref. [34] and for PYT1.

To gain insight into the physiological role of the dicarboxylate carrier, we generated a yeast mutant lacking its gene. First, it was shown that there is no other mitochondrial transport system capable of replacing the dicarboxylate carrier [47]. Swelling experiments in isoosmotic ammonium salts performed with mitochondria isolated from the wild-type and deletion strains as well as from the deletion strain where the dicarboxylate carrier had been reintroduced ectopically, showed that only the mitochondria from the deletion strain were incapable of swelling in the presence of ammonium malate or succinate. Subsequently, it was found that in synthetic minimal medium the deletion strain did not grow on ethanol or acetate as the sole carbon source, but it was viable on other fermentative or nonfermentative carbon sources, including pyruvate and lactate [47]. It is notable that addition of 1.0 mM aspartate, which alone had no effect, restored completely the growth of the mutant cells on acetate or ethanol. Growth of the deletion strain on acetate or ethanol was restored also by addition of low concentrations of oxaloacetate, glutamate, oxoglutarate, citrate and fumarate, but not leucine and lysine, which are unable to generate Krebs cycle intermediates [47]. These findings indicate that the primary function of the dicarboxylate carrier is to transport cytoplasmic dicarboxylates into the mitochondrial matrix, fulfilling an essential anaplerotic role for the Krebs cycle. Therefore, in wild-type *S. cerevisiae* growing on acetate or ethanol, the dicarboxylate carrier catalyzes the import of succinate into mitochondria in exchange for internal phosphate (Fig. 2). Since the latter is recycled back into mitochondria by the phosphate carrier, the combined activity of the dicarboxylate and phosphate carriers leads to a net uptake of succinate. The conversion of succinate to fumarate and subsequently to oxaloacetate within the mitochondria allows the oxidation of acetyl-CoA produced from acetate or ethanol. Also, it triggers the activity of the succinate/fumarate transporter, Sfc1p (see Section 2.2.2 and Fig. 2). In contrast to the *Δdic1*

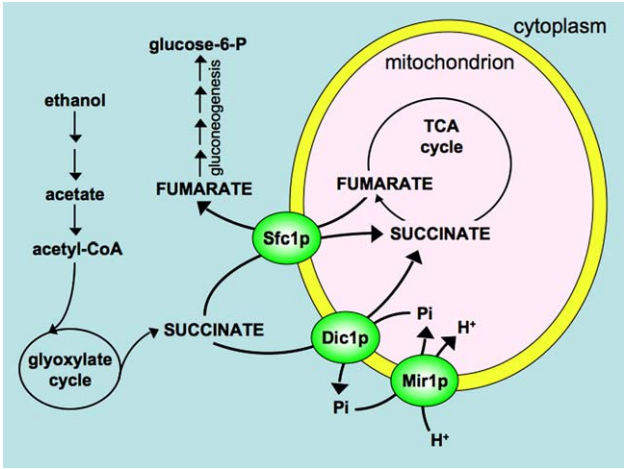

Fig. 2. Roles of the mitochondrial dicarboxylate carrier (Dic1p) and the mitochondrial succinate/fumarate carrier (Sfc1p) on the growth of yeast cells on ethanol or acetate as the sole carbon source. Abbreviations: TCA cycle, tricarboxylic acid cycle; Mir1p, mitochondrial phosphate carrier; Pi, phosphate.

strain, the lack of growth of the *Δsfc1* mutant strain on acetate or ethanol was not restored by addition of compounds that generate intramitochondrial Krebs cycle intermediates [48], in agreement with the conclusion that Sfc1p does not play an anaplerotic role on acetate/ethanol but is necessary for gluconeogenesis. Consistently, expression of the dicarboxylate carrier was repressed by ethanol or acetate, whereas other mitochondrial carriers (including the succinate/fumarate carrier) were not [47]. Clearly, a lower activity of the dicarboxylate carrier favours the utilisation of cytosolic succinate by the succinate/fumarate carrier and thereby the gluconeogenic pathway.

To isolate the cDNA of the human Dic1p ortholog, first we screened the available *Caenorhabditis elegans* sequences with the sequence of Dic1p because the mitochondrial carriers in yeast and man are too distant to allow the mammalian proteins to be cloned on the basis of the yeast sequences. The most closely related protein in *C. elegans* (named K11G12.5) shared 37% identical amino acids with the yeast Dic1p. The reconstituted K11G12.5 protein was shown to be a dicarboxylate carrier by its transport properties. Then, the sequence of the *C. elegans* dicarboxylate carrier was used to isolate two partial overlapping murine ESTs (AA199557, AA041737), which encoded a fragment of a protein with 47% identity with the *C. elegans* dicarboxylate carrier. Finally, these clones were used to isolate full-length mouse, rat and human cDNAs. The encoded proteins are about 95% identical to each other [49,50], and the rat homolog was shown to be a dicarboxylate carrier by transport assays [49]. Recently, also the dicarboxylate carrier of *A. thaliana* was identified by transport assays of the recombinant protein (L. Palmieri et al., unpublished data).

2.1.2. The oxaloacetate/sulfate carrier Oac1p (YKL120w)

The sequence of the yeast YKL120w protein is the second closest to that of the bovine oxoglutarate carrier. It shares 29% and 27% identical amino acids with the bovine oxoglutarate carrier and yeast dicarboxylate carrier, respectively. Therefore, the bacterially expressed and reconstituted YKL120w protein was tested for the transport of various substrates of the dicarboxylate and oxoglutarate carriers. The main substrates of the oxoglutarate and dicarboxylate carriers (oxoglutarate, malate, succinate and phosphate) were transported very poorly, whereas oxaloacetate and sulfate were transported very efficiently by this protein, now named Oac1p [23]. Oac1p catalyzed both unidirectional transport and counter-exchange of substrates. The unidirectional transport of substrates is a co-transport of substrates and H^+, since both the influx and efflux of oxaloacetate or sulfate in and out of the proteoliposomes were dependent on the transmembrane pH gradient. Also, mitochondria from the wild-type but not the *Δoac1* strain swelled in isoosmotic ammonium oxaloacetate or ammonium sulfate solution, whereas both types of mitochondria swelled in ammonium phosphate, proving that the mitochondrial membrane was intact [23].

Because *S. cerevisiae* pyruvate carboxylase is a cytoplasmic protein, one of the physiological roles of the oxaloacetate carrier is to import oxaloacetate into mitochondria at the expense of the proton motive force. This conclusion is supported by the higher transcript level of Oac1p in cells grown in synthetic medium compared to cells grown in rich medium [51]. Yeast cells lacking *OAC1* were able to grow on all fermentable and nonfermentable carbon sources [23]. It is likely that in the absence of the oxaloacetate carrier, oxaloacetate is converted to malate in the cytoplasm and malate enters the mitochondria via the dicarboxylate carrier. A role in anaplerosis for both Oac1p and Dic1p is consistent with the failure of the *Δoac1Δdic1* double mutant to grow on all nonfermentable carbon sources [23].

2.1.3. The oxodicarboxylate carriers Odc1p and Odc2p (YPL134c and YOR222w)

One of our primary aims was to identify the yeast counterpart of the bovine oxoglutarate carrier. However, the yeast proteins with the closest sequence to the bovine oxoglutarate carrier were identified to be the dicarboxylate and oxaloacetate carriers (see Sections 2.1.1 and 2.1.2). The sequences of two other unknown yeast members of the mitochondrial carrier family (Odc1p and Odc2p) were 61% identical to each other and they were both 25% identical to the bovine OGC. Their transport and kinetic properties showed that they are isoforms of a novel mitochondrial transporter named Odc1p and Odc2p. They transport oxoadipate and oxoglutarate with high efficiency and, to a lesser extent, the corresponding dicarboxylates and malate by a counter-exchange mechanism [24]. Odc1p and Odc2p are different from the mammalian oxoglutarate carrier (OGC) in sequence identity (25%), in exhibiting low stereospecificity for L- and D-malate and in transporting C_5–C_7 (oxo)dicarboxylates and, although with low affinity, tricarboxylates. In contrast, OGC transports C_3–C_5 (oxo)dicarboxylates best and does not transport tricarboxylates at all.

The expression of isoform 1 is strongly repressed by glucose and galactose, whereas isoform 2 is expressed on all carbon sources that were tested [24]. Therefore, it is likely that Odc1p is the major carrier isoform under respiratory conditions and that Odc2p is the prevailing isoform in the presence of glucose and possibly in anaerobiosis.

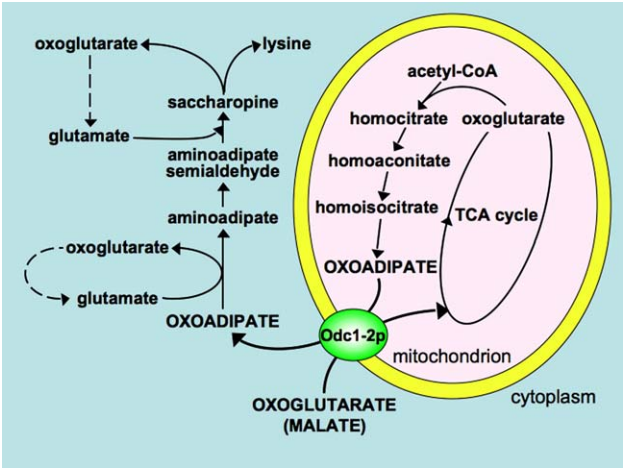

Fig. 3. Role of the mitochondrial oxodicarboxylate carrier (Odc1p–2p) in the synthesis of lysine in yeast. Abbreviations: TCA cycle, tricarboxylic acid cycle.

An important physiological role of the oxodicarboxylate carrier in yeast is to export oxoglutarate (in exchange for malate) to the cytosol, where it is required for nitrogen assimilation by glutamate dehydrogenase, glutamate synthase and glutamine synthase, which are all cytosolic enzymes in yeast. The oxodicarboxylate carrier-mediated exchange between intramitochondrial oxoglutarate and cytosolic malate is required also for the yeast malate/aspartate shuttle (see Section 2.1.5). Furthermore, in yeast, lysine is synthesized via the aminoadipate pathway, whereby oxoadipate is produced in the mitochondria and aminoadipate is converted into lysine in the cytosol. Therefore, it appears that another function of the oxodicarboxylate carrier is probably to export oxoadipate (in exchange for oxoglutarate or malate) from the mitochondria to the cytosol for biosynthesis of lysine (Fig. 3). However, as shown by studies of a prototrophic double deletant, the two isoforms Odc1p and Odc2p are not essential for the synthesis of lysine, indicating a redundant transport system for oxoadipate [52].

As described for the dicarboxylate carrier, the nematode and fruit fly orthologs were used to bridge between the yeast and human orthologs. Also the transport specificity, inhibitors and kinetic parameters of the human oxodicarboxylate carrier (ODC, encoded by SLC25A21) were characterized [53]. Like the yeast protein, the human ODC transports oxoadipate and oxoglutarate with high affinity, but it does not transport malate at all. The physiological function of ODC is to catalyze the uptake of oxoadipate into the mitochondria, where oxoadipate dehydrogenase is found, thus performing a central function in the catabolism of lysine, hydroxylysine and tryptophan. Oxoglutarate is exchanged for oxoadipate by ODC and in the cytosol is required for the first step of lysine catabolism (which converts lysine and oxoglutarate into saccharopine). Consistent with its central role in cell metabolism, ODC is expressed in all tissues that were analyzed [53].

2.1.4. The carnitine carrier Crc1p (YOR100c)

The sequence of the protein product of ORF YOR100c is 29% identical to that of the rat carnitine carrier [7] which is its closest relative. The over-expressed protein was shown to be a carnitine carrier named Crc1p [25]. It transported carnitine, acetylcarnitine, propionylcarnitine with high efficiency and, to a much lower extent, medium- and long-chain acylcarnitines. In contrast to mammals, where only very-long-chain fatty acids are shortened in peroxisomes, in *S. cerevisiae* the β-oxidation of fatty acids is restricted to peroxisomes where they are oxidized to acetyl-CoA, which is converted to acetylcarnitine by the peroxisomal carnitine acetyltransferase Cat2p. Therefore, the main physiological function of the yeast mitochondrial carnitine carrier is to import acetylcarnitine (not medium- and long-chain acylcarnitines) into mitochondria in exchange for free carnitine (Fig. 4). This conclusion is consistent with the co-regulation by oleate of the *CRC1* and *CAT2* genes. *CAT2* encodes carnitine acetyltransferase that is found in both mitochondria and peroxisomes and accounts for more than 95% of the total carnitine acetyltransferase activity in yeast [54]. In mammals, the carnitine carrier catalyzes the entry of acylcarnitines mainly

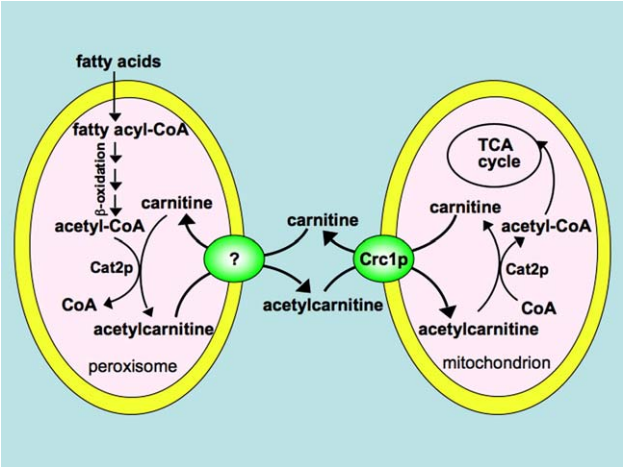

Fig. 4. Role of the mitochondrial carnitine carrier (Crc1p) in the oxidation of fatty acids in yeast. Abbreviations: Cat2p, carnitine acetyltransferase; TCA cycle, tricarboxylic acid cycle. The question mark indicates a still unknown transporter that catalyzes the exchange between acetylcarnitine and free carnitine in the peroxisomal membrane.

of medium and long chains [55,56] in exchange for free carnitine into the mitochondrial matrix, where acyl groups are oxidized. The differences between the substrate specificities of the carnitine carriers from *S. cerevisiae* and mammals reflect the different subcellular compartmentalization of fatty acid metabolism in these organisms. Therefore, the mammalian carrier appears to have evolved to bind and transport medium- and long-chain fatty acids with high efficiency.

In addition to being involved in the complete oxidation of fatty acids, Crc1p also participates in the oxidation of ethanol, which is converted to acetate and then activated to acetyl-CoA in the cytosol (see Fig. 2). It is likely that transfer of the acetyl moiety to L-carnitine is performed by Yat1p associated with outer mitochondrial membrane and/or by cytosolic Yat2p. Expression of these carnitine acetyltransferases is induced by ethanol and acetate but not oleate. Moreover, transport of propionylcarnitine into mitochondria by Crc1p is needed for propionate metabolism which, in *S. cerevisiae*, occurs via the methylcitrate pathway inside the mitochondria [57].

2.1.5. The aspartate/glutamate carrier Agc1p (YPR021c)

Two related human proteins, namely aralar1 and citrin, were identified as isoforms of the mitochondrial aspartate/glutamate carriers (AGC1 and AGC2, encoded by SLC25A12 and SLC25A13, respectively) [41]. They belong to a subfamily of calcium-binding mitochondrial carriers with a characteristic structure consisting of four EF-hand calcium-binding motifs in their N-terminus regions fused to a typical mitochondrial carrier [58,59].

The product of the yeast gene YPR021c was identified by the "similarity approach" as the yeast ortholog of human AGCs and was named Agc1p. It has no N-terminal EF-hand motifs, but the C-terminal domain of Agc1p is 44% and 43% identical, respectively, to the corresponding domains of AGC1 and AGC2. The transport properties of the C-terminal part of Agc1p indicated that it is an aspartate/glutamate carrier that catalyzes an

electrophoretic exchange of negatively charged L-aspartate for electroneutral H^+-compensated L-glutamate [26]. However, the yeast protein differed markedly from the mammalian AGCs in its ability to catalyze the uniport of aspartate, and especially glutamate, in addition to the aspartate/glutamate exchange [26].

In mammalian mitochondria, AGC plays a key role in the malate/aspartate NADH shuttle which transfers reducing equivalents from the cytosol to the mitochondrial matrix for electron transport. The enzymes required to perform this shuttle function are all present in yeast, but the oxidation of cytosolic NADH by external NADH dehydrogenase and the absence of an NADH: ubiquinoneoxidoreductase coupled to the generation of the proton motive force, raises questions about the role of the NADH shuttle in yeast.

To gain insight into the physiological role of Agc1p, the *AGC1* gene was deleted from two *S. cerevisiae* strains (W303 and YPH499). These null mutants did not grow in minimal synthetic medium supplemented with oleic acid or acetate, but they were viable in medium supplemented with other nonfermentable carbon sources including ethanol [26]. The most likely explanation for this phenotype is that Agc1p plays a role in the malate/aspartate shuttle, and this role is critical for growth on acetate and fatty acids (Fig. 5). When yeast is grown on ethanol, the ethanol-acetaldehyde and glycerol-3-phosphate shuttles operate [60,61] and the malate/aspartate shuttle and Agc1p are not required. Yeast strains lacking other components of the malate/aspartate shuttle, such as mitochondrial malate dehydrogenase, Odc1p and Odc2p [52,62], have a phenotype similar to the Δ*agc1* strain, supporting this interpretation.

Metabolic profiling by NMR analysis following incubation with [2-^{13}C]acetate indicated that Agc1p imports glutamate into the mitochondria, in agreement with its ability to catalyze the unidirectional transport of glutamate. Indeed, in the deletant strain the levels of valine, leucine and ornithine, and the incorporation of [2-^{13}C] into alanine (which have a mitochondrial transamination reaction for their synthesis), were decreased [26]. Because these yeast strains are auxotrophic for leucine, the reduction in this amino acid in the *Δagc1* strain relative to wild-type cells was surprising [26]. Probably in *Δagc1* mitochondria leucine (imported by an unidentified mitochondrial branched-chain amino acid carrier) replaces glutamate as the source of amino groups in transamination reactions [63,64].

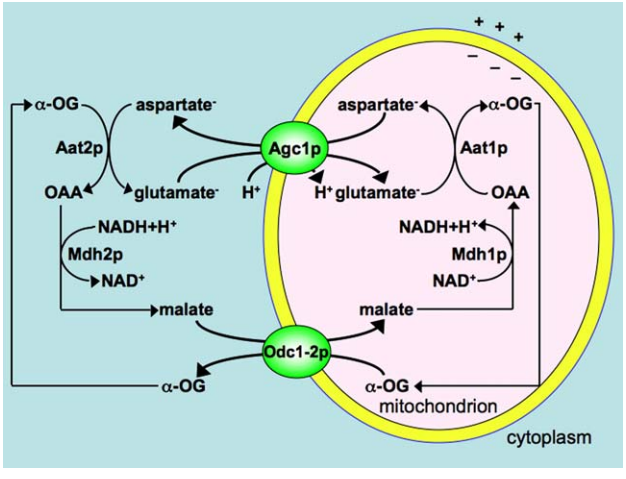

Fig. 5. Role of the mitochondrial aspartate/glutamate carrier (Agc1p) and oxodicarboxylate carrier (Odc1p and Odc2p) in the malate/aspartate shuttle. Abbreviations: Aat1p, mitochondrial aspartate aminotransferase; Aat2p, cytosolic aspartate aminotransferase; Mdh1p, mitochondrial malate dehydrogenase; Mdh2p, cytosolic malate dehydrogenase; α-OG, 2-oxoglutarate; OAA, oxaloacetate.

In contrast to the human AGC, Agc1p does not appear to be important for supplying aspartate to the cytosol. In *S. cerevisiae*, pyruvate carboxylase is located in the cytosol making transport of aspartate out of the mitochondria unnecessary. Furthermore, as *Δagc1* cells grow on ethanol, Agc1p is not needed for gluconeogenesis, and the levels of argininosuccinate and arginine were maintained in Agc1p null mutant cells [26].

2.2. Identification of carriers using genetic and metabolic information

The identification of the substrates of many yeast carriers was based mainly on genetic and metabolic information. In some cases, hints about potential substrates were found in the literature, whereas in other instances the phenotypes of yeast deletion strains provided helpful clues.

2.2.1. The ornithine carrier Ort1p (YOR130c)

Yeast cells with mutations in *ORT1* (ORF, YOR130c) grow poorly in the absence of arginine [65]. The biosynthesis of arginine in *S. cerevisiae* requires five enzymes in the mitochondrial matrix, which produce ornithine from imported glutamate, and three enzymes in the cytosol to convert ornithine exported from the mitochondria into arginine. Our studies of Ort1p showed that it is an ornithine carrier that catalyzes either ornithine transport in exchange for protons or an ornithine/ornithine exchange [27]. It also transports arginine and lysine, though less efficiently, but no other amino acid. Thus, the main physiological role of the yeast ornithine carrier is to transport ornithine in exchange for protons at the expense of the proton motive force, generated by electron transport, from the mitochondrial matrix to the cytosol where it is converted to arginine (Fig. 6). In addition, at high cytosolic concentrations of arginine or lysine, Ort1p catalyzes the entry of arginine or lysine into mitochondria in exchange for ornithine. In the mitochondrial matrix, arginine and lysine are consumed in protein synthesis, and arginine inhibits the first two enzymes of its biosynthetic pathway in a feedback mechanism.

Using the yeast sequence, two isoforms of the human ornithine carrier were identified. They are ORC1 encoded by SLC25A15 [66] and ORC2 encoded by SLC25A2 [67]. In contrast to the yeast ortholog, both human isoforms transport citrulline as well as ornithine, lysine and arginine; ORC2 also transports histidine either by substrate counter-exchange or, to a lesser extent, by exchange for H^+. A primary physiological function of human ORC is to link the activities of urea cycle enzymes in the cytosol to others in the mitochondrial matrix [1,68,69]. Defects in human ORC1 are responsible for the hyperornithinemia–hyperammonemia–homocitrullinemia (HHH)syndrome [66,67]. In patients with HHH syndrome,

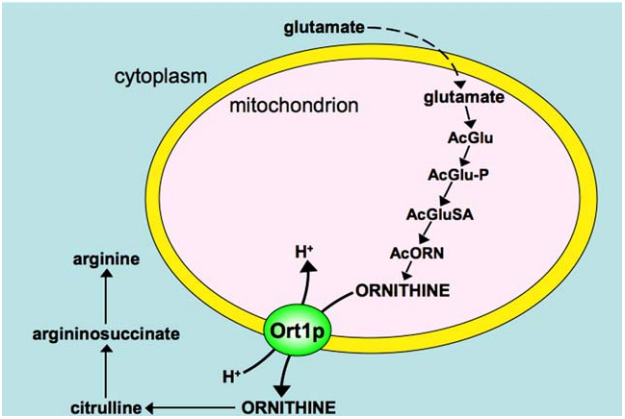

Fig. 6. Role of the mitochondrial ornithine carrier (Ort1p) in the biosynthesis of arginine in yeast. Abbreviations: AcGlu, *N*-acetyl-L-glutamate; AcGlu-P, *N*-acetyl-L-glutamyl-5-phosphate; AcGluSA, *N*-acetyl-L-glutamate-5-semialdehyde; and AcORN, *N*-acetyl-ornithine. The import of glutamate into mitochondria, indicated by a dashed arrow, may be catalyzed by Agc1p (see Section 2.1.5), although there is no direct evidence.

ORC1 is inactive, whereas ORC2 is fully active and compensates partially for the defective ORC1 [67]. Hence, the phenotype of HHH patients is generally milder than those associated with any other defects in the urea cycle. Furthermore, when dietary arginine content is low, and/or in tissues where the activity of arginase is negligible, the ornithine produced from glutamine and glutamate in mitochondria can be exported to the cytosol (in exchange for H^+) to support polyamine biosynthesis and other functions [1,70]. Two distantly related homologs in *A. thaliana*, *BAC1* and *BAC2*, were identified as mitochondrial carriers for basic amino acids from their ability to complement the yeast *ort1* mutant [71,72] and their transport properties assayed upon reconstitution into liposomes [39,71].

2.2.2. The succinate-fumarate carrier Sfc1p (YJR095w)

Yeast cells lacking *SFC1* cannot grow on ethanol or acetate as the sole carbon source [73]. Normally, they are converted to acetyl-CoA which is fed into the glyoxylate and tricarboxylate cycles. As one of the main products of the glyoxylate pathway, succinate is produced from isocitrate in the cytosol and because succinate dehydrogenase is accessible to succinate only from the mitochondrial matrix side, cytosolic succinate must be imported into mitochondria. The transport of succinate and fumarate by recombinant Sfc1p proceeds via a strict counter-exchange mechanism [28], and the fumarate exported to the cytosol in exchange for succinate is converted first to malate and then to oxaloacetate, which enters gluconeogenesis. Therefore, Sfc1p is essential for gluconeogenesis from acetate and ethanol (Fig. 2). This conclusion is substantiated by the finding that *SFC1* is co-regulated with two key enzymes of the gluconeogenic pathway [74]. Furthermore, intermediates entering the tricarboxylic acid cycle are unable to restore the growth of *Δsfc1* cells on ethanol or acetate [48] and so a potential anaplerotic role for this carrier is excluded. Clearly, when sufficient Krebs cycle intermediates are provided by Dic1p in the presence of ethanol and acetate (see Sections 2.1.1), *S. cerevisiae* cells direct the carbon flux to synthetic pathways via Sfc1p.

2.2.3. The S-adenosylmethionine carrier Sam5p (YNL003c)

Yeast cells lacking the gene now called *SAM5* (ORF YNL003c formerly known as PET8) cannot grow on non-fermentable carbon sources such as glycerol and acetate [75]. We found that yeast cells lacking this gene are unable to grow in minimal synthetic medium supplemented with the fermentable substrate glucose or galactose not only in the absence of biotin (like wild-type cells) but also (in contrast to wild-type cells) after the addition of the biotin precursor dethiobiotin [29]. This observation was crucial for identifying the function of Sam5p, since dethiobiotin is converted to biotin by biotin synthetase (Bio2p), which was known to be located inside the mitochondria and require SAM [76]. It could be that Sam5p was required for the uptake of dethiobiotin or for the export of biotin, but direct transport assays in reconstituted liposomes eliminated this possibility. Alternatively, the biotin auxotrophy of the *Δsam5* strain might have been due to an inadequate supply of SAM to the mitochondrial matrix, thereby compromising the conversion of dethiobiotin to biotin. Our studies on Sam5p showed that it is a SAM carrier that catalyzes the uptake of SAM both unidirectionally and in exchange for internal SAM, or, although less efficiently, for internal *S*-adenosylhomocysteine (SAHC) and two non-physiological structurally related compounds, *S*-adenosylcysteine and adenosylornithine (sinefungin) [29]. In the presence of internal 5′-deoxyadenosine, adenosine, methionine, cysteine, ornithine, AMP, cAMP, thiamine pyrophosphate (ThPP), NMN, FMN, oxoglutarate, citrate and many other compounds, the uptake of SAM into proteoliposomes was very similar to the uptake in the absence of an internal substrate (with sodium chloride present), in agreement with the ability of Sam5p to also catalyze uniport.

Therefore, a primary function of Sam5p is to transport SAM, made by the cytosolic synthetases, Sam1p and Sam2p, into the mitochondria, where it is required for the synthesis of both biotin and lipoate (Fig. 7). Additionally, Sam5p catalyzes the exchange of cytosolic SAM for mitochondrial SAHC, which is the by-product of DNA, RNA and protein methylation by SAM. Notably, *S*-adenosylhomocysteine hydrolase (Sah1p) is in the cytosol (see ref. [29] and Fig. 7).

Strong evidence for the role of Sam5p was obtained by expressing the SAM synthetase, Sam1p, inside the mitochondria in *Δsam5* strains. Under these conditions, both the petite phenotype on nonfermentable substrates and the auxotrophy for biotin on fermentable substrates were suppressed [29]. These results clearly indicate that the growth defects of *Δsam5* cells are caused by a shortage of SAM in the mitochondria.

Starting from the yeast ortholog, the human mitochondrial SAM carrier (SAMC encoded by the SLC25A26 gene) was identified from its transport properties in the reconstituted system [36]. Unlike Sam5p, SAMC catalyzed only counter-transport and exhibited a lower transport affinity (Km) for SAM. Recently, also the SAM carrier of *A. thaliana* was identified by transport assays of the recombinant protein (L. Palmieri et al., unpublished data).

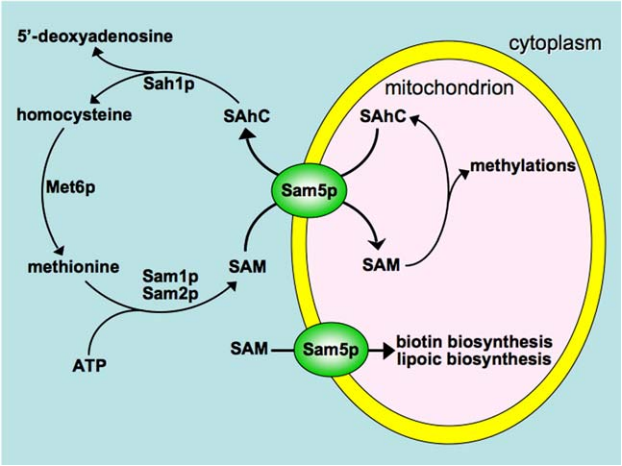

Fig. 7. Role of the mitochondrial transporter for *S*-adenosylmethionine (Sam5p) both in the biosynthesis of biotin and lipoic acid and in the methylation reactions of mtDNA, mtRNA and mitochondrial proteins. Abbreviations: SAM, *S*-adenosylmethionine; SAhC, *S*-adenosylhomocysteine; Sah1p, *S*-adenosylhomocysteine hydrolase; Met6p, methionine synthase; Sam1p and Sam2p, *S*-adenosylmethionine synthetase.

2.2.4. The thiamine pyrophosphate carrier Tpc1p (YGR096w)

Thiamine pyrophosphate (ThPP) is one of several co-factors required for mitochondrial metabolism. Ascorbate, pyridoxine, and hydroxycobalamine (see 30 for references) diffuse through the mitochondrial membrane, whereas ThPP, which is synthesized in the cytosol, does not [77]. At least three enzymes in yeast mitochondria require ThPP, namely acetolactate synthase (ALS), pyruvate dehydrogenase (PDH) and oxoglutarate dehydrogenase (OGDH).

The identification of the YGR096w gene depended on the discovery that yeast cells lacking this gene cannot grow in the absence of thiamine in synthetic minimal medium supplemented with glucose or galactose (but do grow with nonfermentable carbon sources), and that normal growth was restored by adding branched-chain amino acids [30]. Thus, the activity of ALS, which catalyzes the first step in branched-chain amino acid biosynthesis, was affected severely in the YGR096w null mutant by a shortage of mitochondrial ThPP. Because Tpc1p is the closest yeast relative (with 25% sequence identity) to the human deoxynucleotide carrier (DNC encoded by SLC25A19) [78], and isotopically labeled ThPP is not commercially available, the substrate specificity of the reconstituted protein was examined by measuring the uptake of [α-^{35}S]dATP into proteoliposomes that had been pre-loaded with ThPP [30]. Tpc1p transported dATP in exchange for ThPP and thiamine monophosphate (ThMP) and, to a lower extent, (deoxy) nucleotides. The latter compounds were transported according to the following order of efficiency: NMP>NDP>NTP, but nucleosides, purines and pyrimidines were not. Tpc1p catalyzed both uniport and exchange of substrates including ThPP (measured enzymatically). In addition, the proton gradient imposed across the proteoliposomal membrane stimulated the unidirectional transport of dATP or ThPP markedly without influencing the dATP/dATP exchange, suggesting that dATP and ThPP are transported by the reconstituted protein together with H^+ or in exchange for OH^-. It was also shown that Tpc1p localizes to mitochondria [30].

Based on these results, it was concluded that a primary function of Tpc1p is probably to catalyze the uniport uptake of ThPP into the mitochondria, where it is required for ALS, PDH and OGDH activity (Fig. 8). In addition, Tpc1p is necessary to catalyze the exchange between cytosolic ThPP and intramitochondrial ThMP, since ThPP is hydrolyzed in the mitochondrial matrix by ThPPase activity (Fig. 8), as shown in rat liver [79] and *S. cerevisiae* [30].

The following observations confirm that Tpc1p is the mitochondrial ThPP transporter. First, in *Δtpc1* cells grown in minimal synthetic medium supplemented with fermentable carbon sources, with or without thiamine, the amount of ThPP in mitochondria was about 8 times lower relative to wild-type cells [30]. Second, in *Δtpc1* cells the level of ThPP was restored almost to the wild-type level by ectopic expression of the deleted gene [30]. Third, in *Δtpc1* cells the activities of ALS and OGDH in mitochondria were several-fold lower than in mitochondria from wild-type cells, and their activities were restored almost completely by the addition of ThPP to assay solutions [30].

2.2.5. The adenine nucleotide carrier Ant1p (YPR128c)

The peroxisomal adenine nucleotide carrier encoded by the YPR128c gene, now called *ANT1*, is an integral protein in yeast peroxisomal membranes [31]. It is essential for growth on medium-chain fatty acids as the sole carbon source [31], and because its promoter region contains an oleate response element, expression increases in the presence of fatty acids [31], indicating clearly that Ant1p is involved in fatty-acid oxidation. Moreover, β-oxidation of fatty acids in yeast is restricted to peroxisomes, and medium-chain fatty acids are activated by the peroxisomal acyl-CoA synthetase Faa2p in an ATP-dependent way.

Therefore, yeast Ant1p appeared to be responsible for one of the following transport reactions: uptake of medium-chain fatty

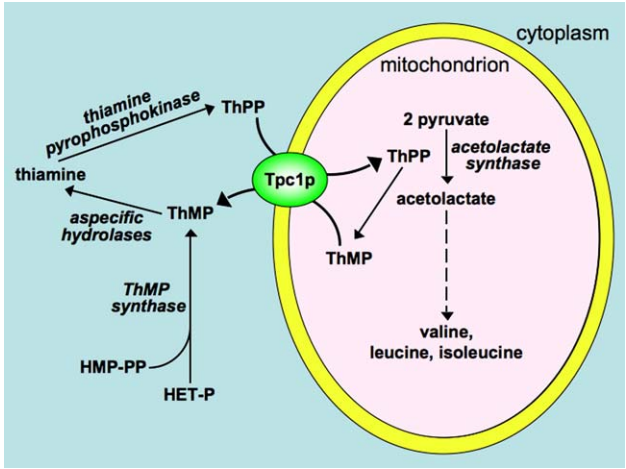

Fig. 8. Role of the mitochondrial thiamine pyrophosphate carrier (Tpc1p) in the biosynthesis of branched chain amino acids in yeast. Abbreviations: ThPP, thiamine pyrophosphate; ThMP, thiamine monophosphate; HET-P, hydroxyethylthiazole phosphate; HMP-PP, hydroxymethylpyrimidine pyrophosphate.

acids, uptake of ATP (required for their activation), export of AMP (one of the products of the activation reaction), and uptake of co-factors (coenzymes) required for the functioning of enzymes of β-oxidation. The reconstituted Ant1p transported ATP, ADP, AMP and, to a lower extent, the corresponding deoxynucleotides [31]. It differs from the mitochondrial ADP/ATP carrier by transporting AMP, by being unaffected by inhibitors of the ADP/ATP carrier, such as carboxyatractyloside and bongkrekic acid, and by being only 13–16% identical with the isoforms of the yeast ADP/ATP carrier. The physiological role of Ant1p is to transport cytosolic ATP into the peroxisomal lumen in exchange for AMP generated in fatty acid activation (Fig. 9). Ant1p was the first peroxisomal membrane protein proven to perform a transport function. It is also the first biochemically characterized member of the mitochondrial carrier family not to be found in mitochondria. The human ortholog was identified with the sequence of Ant1p [80].

2.2.6. The pyrimidine nucleotide transporter Pyt1p, known as Rim2p (YBR192w)

Although the protein encoded by *PYT1* (*RIM2*, YBR192w) belongs to the mitochondrial carrier family, it differs from most members of the mitochondrial carrier family insofar as its N-terminal region, the matrix loop between transmembrane segments I and II and the cytosolic loop between transmembrane segments IV and V of Pyt1p (Rim2p) are longer than usual. Another feature of Pyt1p is the presence of a tryptophan (PI*W*LIK) instead of an acidic residue in the signature motif of the second repeat.

Rim2p (Pyt1p) was first described by Van Dyck et al. [81] as a multicopy suppressor localized in mitochondria that rescues partially the growth defect of either the *PIF1* or *MRS2* null mutant (Pif1p is a DNA helicase required for mtDNA maintenance [82] and Mrs2p is a Mg^{2+} transporter required in mitochondrial RNA splicing [83,84]). Furthermore, it was found that deletion of *RIM2* causes loss of mtDNA and lack of growth on nonfermentable carbon sources [81].

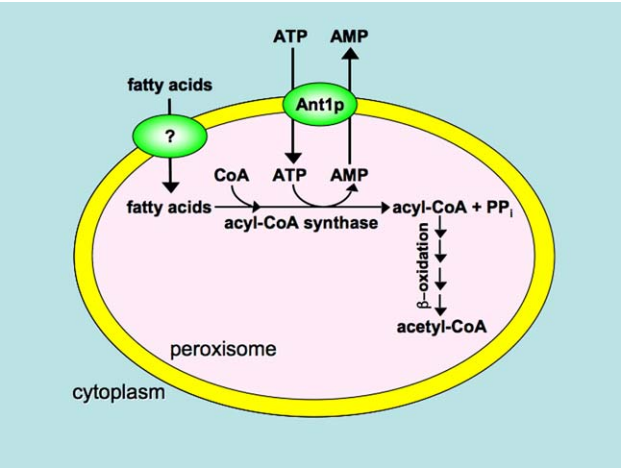

Fig. 9. Role of the peroxisomal adenine nucleotide transporter (Ant1p) in the β-oxidation of fatty acid in yeast. Abbreviation: PPi, pyrophosphate. The question mark indicates an unknown mechanism of medium-chain fatty acid import into peroxisomes.

A number of observations suggested that Pyt1p might transport pyrimidine (deoxy)nucleotides. In *S. cerevisiae*, pyrimidine nucleoside diphosphates are synthesized outside mitochondria [76] and nucleoside diphosphate kinase (Ynk1p) and ribonucleotide reductases (Rnr1p–4p) are also found outside the inner mitochondrial membrane [85,86]. (Deoxy) pyrimidine nucleoside triphosphates (Py(d)NTPs) are required inside mitochondria for DNA and RNA synthesis, including the synthesis of RNA primers which are necessary to initiate DNA replication and repair. Therefore, a means to import them into mitochondria must exist. Several proteins had been identified as mitochondrial carriers for purine nucleotides [15–17,31,33] but none of them transported pyrimidine nucleotides. Pyt1p proved to be the mitochondrial transporter for pyrimidine nucleotides [32]. It catalyzes homo- and hetero-exchange (not unidirectional transport) of all pyrimidine (deoxy)nucleotides, and (deoxy) nucleoside di- and triphosphates are transported more efficiently than nucleoside monophosphates; pyrimidine deoxynucleotides are transported to about the same extent as the corresponding nucleotides.

The main physiological role of Pyt1p is to transport PyNTPs and Py(d)NTPs into mitochondria, where they are incorporated into mtRNA and mtDNA, respectively, in exchange for the corresponding NMPs, produced in the catabolism of mitochondrial RNA and DNA (Fig. 10). For thermodynamic reasons, and also because PyNDPs are phosphorylated by the nucleoside diphosphate kinase outside the inner membrane of yeast mitochondria [85], the Pyt1p-mediated import of PyNDPs in exchange for PyNMPs should be relatively minor compared with the uptake of PyNTPs in exchange for PyNMPs. If they were transported into mitochondria in exchange for PyNMPs, PyNDPs would recycle across the membrane by exchanging with external PyNTPs, leading to a net $PyNTPs_{out}/PyNMPs_{in}$ exchange. The human proteins Q96CQ1, Q9BSK2 and NT_009237.17, which are 32%, 29% and 29% identical respectively to Pyt1p, are likely to be orthologs.

2.3. Identification of yeast carriers using phylogenetic analysis

2.3.1. The GTP/GDP carrier Ggc1p (YDL198c)

By phylogenetic analysis of the yeast mitochondrial carriers [12,13] it was found that the YDL198c gene product clusters together with the three isoforms of the ADP/ATP carrier [15,17], with the carriers for thiamine pyrophosphate [30] and coenzyme A [87], and with two proteins (YDL119c and YPR011c) which have not yet been identified. Therefore, the recombinant protein was tested for its ability to transport nucleotides and nucleotide-like molecules. It transported (d) GTP and (d)GDP with high efficiency and high affinity by a strict counter-exchange mechanism [33]. Its Km was about 1 μM for GTP, 5 μM for GDP and 15 μM for dGTP. Therefore, it appears to be a carrier for GTP and GDP and so was named Ggc1p. Its primary function is to catalyze the transport of GTP from the cytosol into mitochondria in exchange for GDP (Fig. 11). This conclusion is supported by the following findings. First, Ggc1p, previously known as Yhm1p, is localized in mitochondria [88]; second, in *S. cerevisiae,* GTP is not

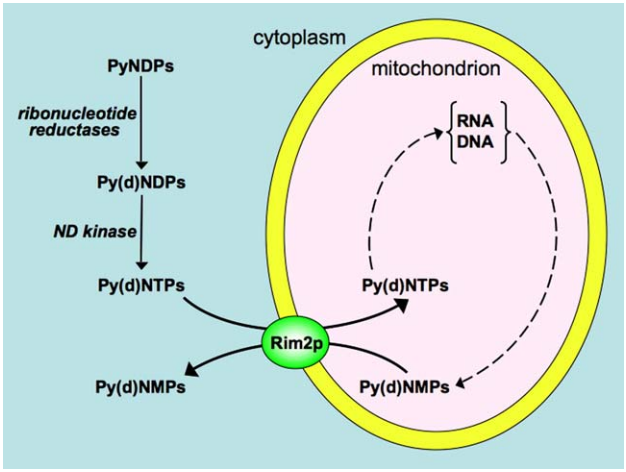

Fig. 10. Role of the mitochondrial pyrimidine nucleotide transporter (Rim2p/Pyt1p) in the synthesis and degradation of mtDNA and mtRNA. Abbreviations: PyNDPs, pyrimidine nucleoside diphosphates; Py(d)NDPs, pyrimidine (deoxy) nucleoside diphosphates; Py(d)NTPs, pyrimidine (deoxy)nucleoside triphosphates; and Py(d)NMPs, pyrimidine (deoxy)nucleoside monophosphates; ND kinase, nucleoside diphosphate kinase.

synthesized in the mitochondrial matrix [85,89]; third, GDP is produced from GTP intramitochondrially in protein synthesis for the formation of the initiation complex and for the elongation of the polypeptide chain, and by the activities of GTP-AMP phosphotransferase and G proteins [90,91]; fourth, Ggc1p operates by an obligatory counter-exchange mechanism [33]; fifth, the GTP/GDP hetero-exchange (but not GTP/GTP and GDP/GDP homo-exchanges) depends on the transmembrane pH gradient and not on the electric potential (i.e., the GTP/GDP exchange is electroneutral H^+-compensated) [33]; sixth, the level of GTP in mitochondria is about 7-fold lower in the *ggc1* null mutant than in the wild-type cells [33]; and seventh, in agreement with its role in the maintenance of mtDNA, the *ggc1* null mutant does not grow on any non-fermentable carbon source and has no mtDNA [33].

GTP is also required in the mitochondrial matrix for the synthesis of mRNA, tRNA, rRNA and for initiation of DNA replication and repair, and dGTP for the synthesis of DNA. In the synthesis of both DNA and RNA pyrophosphate is produced. We do not yet know whether the uptake of GTP and dGTP necessary for these reactions is imported by Ggc1p or by some other carrier, as well as the outcome for pyrophosphate.

2.3.2. The nicotinamide dinucleotide carriers Ndt1p and Ndt2p (YIL006w and YEL006w)

The proteins encoded by YIL006w and YEL006w cluster in the phylogenetic tree of yeast carriers with Rim2p and Flx1p, the transporters of pyrimidine nucleotides [32] and FAD [92], respectively, and so attempts to uncover their functions were predicated on the assumption that they would transport nucleotide-type molecules also. It was found that the gene product of YIL006w transports NAD^+ and various other nucleotides in exchange for NAD^+ [34]. The transport of NAD^+ is highly specific and internal α-NAD^+, NADH, NMN, NAMN and ADP-ribose were very poor counter-substrates. Internal purine nucleotides exchanged with NAD^+ more effectively than pyrimidine nucleotides. Nucleoside monophosphates were transported more effectively than nucleoside di- and triphosphates, and the transport of deoxynucleotides were almost as effective as that of the corresponding nucleotides. As well as conducting exchange of NAD^+, the YIL006w gene product also has a uniport activity for the same substrate [34].

The sequence of the YEL006w gene product is 70% homologous and 55% identical to that of the YIL006w product. For this reason, the gene products of the YIL006w and YEL006w have been named Ndt1p and Ndt2p, respectively [34]. However, the reconstitution of the bacterially expressed YEL006w protein has not succeeded hitherto, and its function has not been demonstrated directly [34]. Both proteins are localized in mitochondria. Their transport function is consistent with the enzymes for NAD^+ biosynthesis being outside the yeast mitochondria [93,94] and with the lack of activity of the two NAD^+- synthesizing enzymes, NAD^+ synthetase and NAMN/NMN adenlylyltransferase in mitochondria [34]. Therefore, NAD^+ required for activities in the mitochondrial matrix must be imported from the cytosol. Hence, the main function of Ndt1p and Ndt2p is to import NAD^+ into the mitochondria by unidirectional transport or by exchange with nucleoside monophosphates, most likely AMP, GMP and their deoxyderivatives, generated in mitochondria by breakdown of RNA and DNA.

The functions of Ndt1p and Ndt2p were confirmed in vivo by measuring the NAD^+ and NADH contents of mitochondria from the wild-type and deletion strains. In both the *Δndt1* and *Δndt2* single deletants, the mitochondrial NAD^+ and NADH contents were lower than in controls, and complementation with the deleted gene restored these levels, demonstrating that both isoforms are involved in transport of NAD^+ into mitochondria. In the double deletion strain, *Δndt1Δndt2*, the NAD^+ content of mitochondria was significantly lower than in mitochondria of the single deletion strains. Complementation of the double deletion strain with only one of the two genes restored the NAD^+ content of mitochondria partially, confirming that both

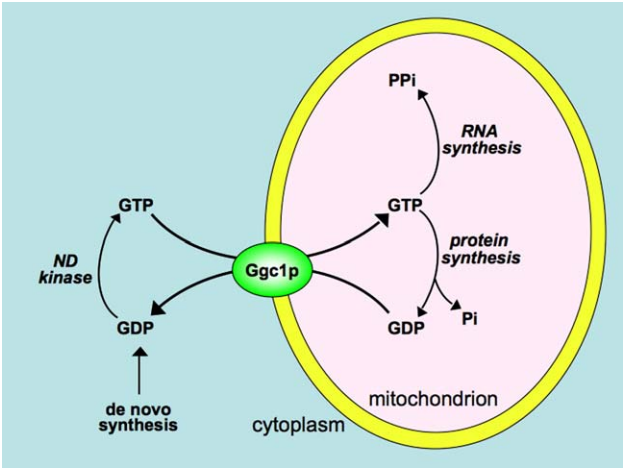

Fig. 11. Role of the mitochondrial GTP/GDP carrier (Ggc1p) in protein synthesis and RNA synthesis in yeast mitochondria. Abbreviations: ND kinase, nucleoside diphosphate kinase; Pi, phosphate; PPi, pyrophosphate.

isoforms are required [34]. The assigned functions of Ndt1p and Ndt2p as NAD^+ carriers are consistent with the activities of two mitochondrial NAD^+-requiring enzymes, pyruvate dehydrogenase and acetaldehyde dehydrogenase, being lower in mitochondria from the single deletant strains, and lower still in the double mutant, than in mitochondria from the wild-type strain. The addition of NAD^+ restored both activities to values similar to those of the wild-type mitochondria, indicating that the reduced activities of these enzymes are caused by a lack of NAD^+ in the mitochondrial matrix [34]. Also, the growth of the *Δndt1Δndt2* double mutant (but not of the single deletants), especially in synthetic minimal medium supplemented with ethanol and other nonfermentable substrates (but not with glucose), was retarded. Normal growth was restored by complementing the double mutant with either of the genes encoding Ndt1p and Ndt2p, demonstrating that the impaired phenotype is caused directly by the absence of one of the two proteins [34]. Clearly, the marked decrease in the mitochondrial contents of NAD^+ and NADH in the double mutant strain delays growth for lack of metabolic energy due to inactivation of NAD^+-dependent enzymes, including acetaldehyde dehydrogenase that catalyzes the rate-limiting step of yeast growth on ethanol [95]. Ndt1p and Ndt2p are the only mitochondrial carriers for NAD^+ yet to be described in any organism. Ndt1p has been reported previously to be the mitochondrial pyruvate carrier in *S. cerevisiae* [96]. This conclusion was based on the observation that among the mitochondria isolated from 18 different *S. cerevisiae* mutants, each lacking an unattributed member of the mitochondrial carrier family, only those from the Δ*ndt1* mutant exhibited no inhibitor-sensitive transport of pyruvate [96]. However, the recombinant and reconstituted Ndt1p did not transport pyruvate either as homo-exchange or as hetero-exchange with NAD^+.

2.4. Other known mitochondrial carriers of yeast

In addition to the 3 isoforms of the ADP/ATP carrier (Aac1-3p), the transporters for phosphate (Mir1p) and citrate (Ctp1p), and the 15 carriers described above, the functions of 6 additional yeast transporters have been identified from various pieces of evidence. They include genetic studies, complementation of deletion strains and transport measurements in mitochondria or submitochondrial particles isolated from null mutants and wild-type strains. These six transporters are the carriers for FAD (Flx1p) [92], CoA (Leu5p) [87], ATP-Mg/phosphate (Sal1p) [97], iron (Mrs3p and Mrs4p) [98,99] and the second isoform of the phosphate carrier (Pic2p) [100]. However, the identities of these carriers must await biochemical confirmation.

3. Concluding remarks

The research approach based on direct assays of the transport activities of recombinant, purified and reconstituted mitochondrial carriers encoded by the genomes of various organisms has proved to be a powerful tool for discovering the transport properties of previously unknown members of the mitochondrial carrier family. More than half of the members of the *S. cerevisiae* mitochondrial carrier family have been identified by direct transport assays, and often the yeast sequences have been used to identify orthologs in other organisms.

A few members of the family in *S. cerevisiae* still remain to be identified. This task may be aided by the recent identification of a common binding site in mitochondrial carriers [101], which may provide a basis for predicting the potential substrates of unknown carriers. The identification of new mitochondrial carriers in yeast and other organisms will continue to shed light on their involvement in metabolic pathways and lead to the discovery of unknown regulatory mechanisms, thereby providing new insights into the physiological roles of mitochondrial carriers in cell metabolism. This research is medically important as defects in some carriers are linked to human diseases [1]. Therefore, the identification of carrier function can help define the molecular basis of a disease and provide an explanation of its symptoms.

The biochemically characterized members of the mitochondrial carrier family transport several types of substrates (carboxylates, oxodicarboxylates, amino acids, nucleotides and co-factors) ranging from protons to NAD^+, which may prove to be the largest molecule transported by a solute carrier protein. They operate with a variety of mechanisms including uniport, symport and antiport; also, the electrical nature of the mitochondrial carrier-mediated transport can be electroneutral, proton compensated or electrophoretic. The models of the three-dimensional structures of the members of the mitochondrial carrier family, together with the wide range of functional data, make them attractive for investigating the catalytic step mechanisms at the molecular level.

Acknowledgments

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