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Yeast Mitochondrial Carriers: Bacterial Expression, Biochemical Identification and Metabolic Significance

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The genome of *Saccharomyces cerevisiae* encodes 35 members of a family proteins that transport metabolites and substrates across the inner membranes of mitochondria. They include three isoforms of the ADP/ATP translocase and the phosphate and citrate carriers. At the start of our work, the functions of the remaining 30 members of the family were unknown. We are attempting to identify these 30 proteins by overexpression of the proteins in specially selected host strains of *Escherichia coli* that allow the carriers to accumulate at high levels in the form of inclusion bodies. The purified proteins are then reconstituted into proteoliposomes where their transport properties are studied. Thus far, we have identified the dicarboxylate, succinate–fumarate and ornithine carriers. Bacterial overexpression and functional identification, together with characterization of yeast knockout strains, has brought insight into the physiological significance of these transporters. The yeast dicarboxylate carrier sequence has been used to identify the orthologous protein in *Caenorhabditis elegans* and, in turn, this latter sequence has been used to establish the sequence of the human ortholog.

KEY WORDS: Mitochondria; transport; overexpression; dicarboxylate carrier; ACR1 gene; succinate–fumarate exchange; ARG11 gene; ornithine carrier; arginine biosynthesis; yeast; metabolism.

INTRODUCTION

The inner membranes of mitochondria contain a family of proteins of related sequence and structure that transport various metabolites across the membrane (Kramer and Palmieri, 1992; Walker, 1992; Palmieri, 1994; Palmieri and van Ommen, 1999). Their amino acid sequences have a tripartite structure, made up of three related sequences about 100 amino acids in length. The repeats of one carrier are related to those present in the others and several characteristic sequence features are conserved throughout the family. Despite extensive functional analysis, chemical modification, and site-directed mutagenesis studies on particular mitochondrial carrier proteins, the mechanism of solute transport is still a major challenge in biomembrane research. The inability to obtain abundant quantities of purified mitochondrial transporters has significantly impeded the acquisition of structural and functional information. A breakthrough was achieved when Fiermonte *et al.* (1993) succeeded in expressing the bovine oxoglutarate carrier at high levels in *E. coli*. The recombinant oxoglutarate carrier accumulated as inclusion bodies in the bacteria, was refolded in the presence of the zwitterionic detergent *N*-dodecanoylsarcosine (sarkosyl) and functionally reconstituted into liposomes. This was the first time that an eukaryotic membrane protein has been overproduced in *E. coli* and renatured. With minor modification, the same procedure was subsequently employed for overexpressing and functionally reconstituting the phosphate (Palmieri, 1994; Wohlrab and Briggs, 1994; Fiermonte *et al.*,

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1998a), the citrate (Xu *et al.*, 1995; Kaplan *et al.*, 1995), and the carnitine carriers (Indiveri *et al.*, 1998). Therefore, the supply of mitochondrial transporters is no longer a problem.

In spite of their close structural relationship, biochemically characterized members of the mitochondrial carrier family handle the most extreme types of substrates, from the smallest, *i.e.*, protons, to one of the largest molecules transported by carrier proteins, *i.e.*, ATP. They operate with various mechanisms, which include uniport, symport, and antiport, electroneutral, proton-compensated or electrophoretic mechanisms. It is thus of major interest to accumulate functional data for different carrier molecules in order to gain insight into how a shared basic protein structure can be adapted to very different functional requirements. Indeed, the complete elucidation of the translocational events occurring through each individual mitochondrial carrier will require both functional and structural analysis. Extensive kinetic and thermodynamic information is accumulating because of functional studies of many mitochondrial carriers in the reconstituted system. This, together with the expected close relationship of their three-dimensional (3-D) structures, makes the mitochondrial carrier family an attractive model for understanding the catalytic mechanism of biological transport at a molecular level.

In this context, bacterial overexpression of genomic sequence-encoding members of the mitochondrial carrier family is a powerful tool for expanding our knowledge on mitochondrial transport. As reviewed here, we have extended our studies on bacterial expression of mammalian mitochondrial transporters to putative members of the mitochondrial carrier family in *Saccharomyces cerevisiae*, as its complete genome sequence is available and genetic manipulation is relatively easy. In this way, we have already identified the function of three *S. cerevisiae* gene products, whose structural and functional properties assigned them to the mitochondrial carrier family. The yeast dicarboxylate carrier sequence has, in turn, been used to identify its distantly related mammalian homologs. The functional information obtained in liposomes reconstituted with the recombinant proteins and (as exemplified here for the dicarboxylate carrier) the use of knockout yeast strains may be useful in investigating the physiological role of the individual transporters and in shedding light on the integration of mitochondria into cell metabolism.

BACTERIAL OVEREXPRESSION OF PUTATIVE YEAST MITOCHONDRIAL CARRIERS

We developed a procedure to overexpress in *E. coli* and to solubilize and reconstitute mitochondrial carrier proteins in active form (Fiermonte *et al.* 1993). There are two main advantages to this technique: (1) the high degree of purity of the expressed transporters in the inclusion bodies; (2) the simple and efficient refolding of the carrier proteins upon solubilization with sarkosyl. Bacterial expression and functional reconstitution into liposomes can be used to identify the function of putative members of the mitochondrial carrier family encoded in the genomes of various organisms. As soon as the genomic sequence of *S. cerevisiae* was completed, it was screened for members of the mitochondrial carrier family based on the sequence features of this protein family. Our analysis revealed 35 putative members upon comparing the proteins encoded in the yeast genome with the sequence of known mammalian carriers with the aid of the BLAST program (Fig. 1; Palmieri *et al.*, 1996; El Moualij *et al.*, 1997; Nelson *et al.*, 1998). They included three isoforms of the ADP/ATP translocase (Adrian *et al.*, 1986; Lawson and Douglas, 1988; Kolarov *et al.*, 1990), and the transport proteins for phosphate (Wohlrab and Briggs, 1994) and citrate (Kaplan *et al.*, 1995).

Our attempts to overexpress the remaining 30 genes, whose function was unknown, showed that nine mitochondrial carrier proteins could not be expressed under the same conditions used for the bovine oxoglutarate carrier (Fiermonte *et al.*, 1993). Although we managed to recover 10–15 mg of the bovine oxoglutarate carrier per liter of bacterial culture (Fiermonte *et al.*, 1993), Miroux and Walker (1996) reported that most of *E. coli* BL21(DE3) cells died shortly after the onset of the oxoglutarate carrier expression. Taking advantage of the oxoglutarate carrier's toxicity, they selected a new *E. coli* strain C41(DE3), a spontaneous mutant of the original host strain in which the expression of the bovine oxoglutarate carrier was no longer toxic. About 200 mg of oxoglutarate carrier per liter of bacterial culture were obtained employing this mutant *E. coli* strain. However, some transporters, including the isoform A of the bovine phosphate carrier (Fiermonte *et al.*, 1998a), proved to be toxic even to C41(DE3). Therefore, the strain C0214(DE3), a mutant of C41(DE3) able to tolerate expression of phosphate carrier isoform A, was isolated following a similar

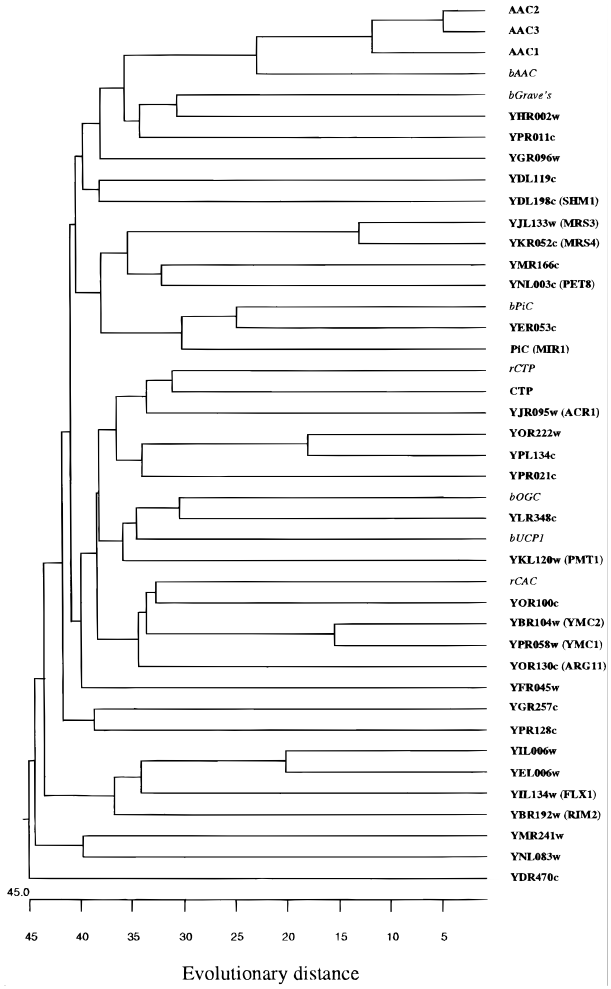

Fig. 1. Phylogenetic tree of the *S. cerevisiae* 35 putative members of the mitochondrial carrier family. The tree was computed using a PAM250 matrix and the PHYLIP package (version 3.5c) developed by Felsenstein (1989). The yeast sequences are indicated in bold type; acronyms are given in brackets. The mammalian sequences used to screen the fungal genome are indicated in italics: *bOGC*, bovine oxoglutarate carrier; *bUCP1*, bovine uncoupling protein 1; *rCTP*, rat citrate carrier; *rCAC*, rat carnitine/acylcarnitine carrier; *bAAC*, bovine ADP/ATP carrier, isoform 1; *bGrave's*, bovine Grave's disease protein; *bPiC*, bovine phosphate carrier, isoform A.

procedure to that used for the isolation of C41(DE3) from BL21(DE3) (Fiermonte and Walker, 1994, unpublished results). With few exceptions, mitochondrial yeast carriers can be expressed in C0214(DE3) cells at high levels. An example is given by the yeast ornithine carrier (Palmieri *et al.*, 1997a) encoded by the YOR130c gene. Only 2 mg of YOR130c gene product per liter of bacterial culture were obtained using BL21(DE3) as host strain (Mayor *et al.*, 1997), whereas 50 mg/L were obtained with the CO214(DE3) strain (Palmieri *et al.*, 1997a). It should also be emphasized that the purity of the mitochondrial carriers overexpressed using the C41(DE3) and CO214(DE3) strains (90–100% of the protein present in the inclusion bodies) is usually much higher than that obtainable using the BL21(DE3) cells. Table I lists all the yeast mitochondrial transporters that have so far been overexpressed in *E. coli*, refolded by the sarkosyl solubilization procedure and functionally reconstituted into liposomes. In our laboratories, three yeast mitochondrial carriers, *i.e.*, the dicarboxylate, the succinate–fumarate, and the ornithine carriers, have been successfully overexpressed and functionally identified. Transport assays were carried out in proteoliposomes reconstituted with the recombinant, purified proteins by the cyclic detergent-removal procedure (Palmieri *et al.*, 1995). In this procedure, mixed micelles containing nonionic detergent, protein, and phospholipids are repeatedly passed through the same column filled with the hydrophobic resin Amberlite XAD-2. This recycling hydrophobic chromatography presents several advantages over the freeze–thaw–sonication procedure (Kasahara and Hinkle, 1977) which has long been used for reconstituting mitochondrial carriers: (1) it allows incorporation of higher amounts of protein; (2) the proteoliposomes are larger and more homogeneous; (3) the ratio between the active volume, *i.e.*, the internal volume of the liposomes containing active

Table I. Yeast Mitochondrial Carriers Overexpressed in *E. coli*, Refolded in the Presence of Sarkosyl, and Functionally Reconstituted[a]

Carrier	Bacterial strain	Expression level(mg/L)
Phosphate carrier[c]	BL21(DE3)	2
Citrate carrier[d]	BL21(DE3)	25[b]
Dicarboxylate carrier[e]	C41(DE3)	25
Dicarboxylate carrier[f]	BL21(DE3)	28[b]
Ornithine carrier[g]	C0214(DE3)	50
Succinate–fumarate carrier[h]	C0214(DE3)	75

[a] Following the procedure originally proposed by Fiermonte *et al.* (1993).
[b] Purity 75%.
[c] From Wohlrab and Briggs (1994).
[d] From Kaplan *et al.* (1995).
[e] From Palmieri *et al.* (1996).
[f] From Kakhniashvili *et al.* 1997).
[g] From Palmieri *et al.* (1997a).
[h] From Palmieri *et al.* (1997b).

carrier, and the total internal volume of the vesicles, is higher; (4) the almost total removal of detergent allows the formation of tighter proteoliposomes with a much lower unspecific permeability. For optimal activity, in the case of the yeast carriers, addition of cardiolipin to the reconstitution mixture is often mandatory.

IDENTIFICATION OF THE YEAST ACR1 GENE PRODUCT AS A SUCCINATE–FUMARATE TRANSPORTER ESSENTIAL FOR GROWTH ON ETHANOL OR ACETATE

Yeast *S. cerevisiae* can grow aerobically on either ethanol or acetate as the sole carbon source. Ethanol is converted to acetate via acetaldehyde, and acetate is activated to acetyl-CoA, which can be fed into the glyoxylate and tricarboxylate cycles. The glyoxylate pathway is essential for the utilization of C-2 compounds (Fernandez *et al.*, 1992). Succinate, one of the principal products of the glyoxylate pathway, is produced in the cytosol from isocitrate (Taylor *et al.*, 1996). As succinate dehydrogenase is accessible to succinate only from the mitochondrial matrix side (Quagliarello and Palmieri, 1968), the succinate produced in the cytosol has to be imported into mitochondria. *Saccharomyces cerevisiae* cells lacking the ACR1 gene are unable to grow on ethanol or acetate as the sole carbon source (Fernandez *et al.*, 1994). The protein encoded by the ACR1 gene (or YJR095w) belongs to the mitochondrial carrier family. The ACR1 gene was overexpressed in *E. coli* (50 mg/L culture) and the gene product was solubilized with sarkosyl, purified, and functionally reconstituted into liposomes (Palmieri *et al.*, 1997b). The expressed ACR1 protein was shown to catalyze an obligatory oxoglutarate–oxoglutarate exchange in the reconstituted system. The substrate specificity of the ACR1 protein was first investigated by measuring the uptake of labeled oxoglutarate into proteoliposomes that had been loaded with a variety of substrates. The highest activities were observed in the presence of succinate and fumarate. To a lesser extent, ^{14}C-oxoglutarate was also taken up in exchange for intraliposomal oxoglutarate and oxaloacetate. In order to obtain further information about the transport properties of the ACR1 protein, we studied the kinetic parameters of transport and the ability of various substrates to act as competitive inhibitors. The recombinant protein displayed K_m values for fumarate and succinate of 0.36 $\pm$ 0.08 mM and 0.69 $\pm$ 0.12 mM, respectively. The K_m values for oxoglutarate and oxaloacetate were substantially higher, *i.e.*, 2.0 $\pm$ 0.11 mM and 1.93 $\pm$ 0.28 mM, respectively; those for L-malate, phosphoenolpyruvate, citrate, and isocitrate were still higher. These data indicate that fumarate and succinate are the physiological substrates of the ACR1 protein. Based on these findings, we proposed that the metabolic significance of the ACR1 protein in *S. cerevisiae* is to transport succinate, produced in the cytosol by the glyoxylate pathway, into the mitochondrial matrix in exchange for fumarate (Palmieri *et al.*, 1997b). Therefore, the ACR1 gene product, *i.e.*, the succinate–fumarate transporter, connects the production of succinate by the glyoxylate cycle in the cytosol with the tricarboxylate acid cycle and electron transfer by complex II (succinate dehydrogenase: ubiquinone reductase) in the mitochondria. Fumarate exported to the cytosol in exchange with succinate by the succinate–fumarate carrier is converted first to malate, and then to oxaloacetate, which is funneled into the gluconeogenic pathway (Fig. 2) and is indispensable for *S. cerevisiae* growth on ethanol or acetate (Gancedo and Serrano, 1989). In line with the proposed role of the succinate–fumarate carrier in gluconeogenesis, ACR1 shares a common upstream activating sequence and a similar temporal pattern of expression (during the diauxic shift from fermentation to respiration) with five enzymes involved either in the utilization of ethanol via the glyoxylate cycle or in the gluconeogenic pathway (DeRisi *et al.*, 1997). Furthermore, Bojunga *et al.* (1998) and Redruello *et al.* (1999) have recently demonstrated that the derepression of ACR1 is dependent on the transcriptional activator Cat8p and, therefore, it is strictly co-regulated with the genes encoding key enzymes of the glyoxylate cycle (isocitrate lyase and malate synthetase) and gluconeogenesis (phosphoenolpyruvate carboxykinase and fructose-1,6-Bisphosphatase).

IDENTIFICATION OF THE *Saccharomyces cerevisiae* GENE ENCODING THE MITOCHONDRIAL DICARBOXYLATE CARRIER

It was suggested that the mitochondrial metabolite transporters, which had been biochemically characterized but not yet sequenced, also belong to the mitochondrial carrier protein family (Kramer and Palmieri, 1992). One example is provided by the dicarboxylate carrier. This transporter was originally identified in

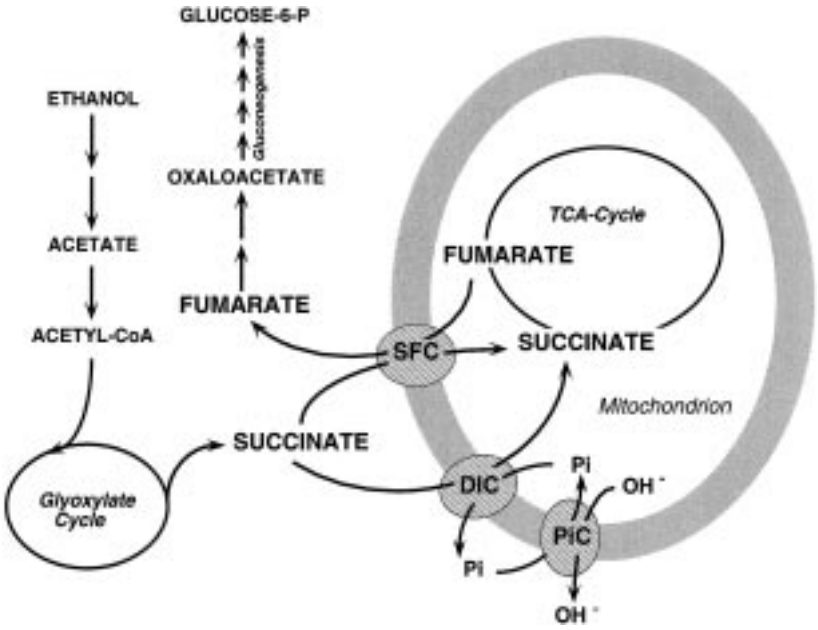

Fig. 2. Pathways involved in succinate metabolism in *S. cerevisiae.* DIC, dicarboxylate carrier; PiC, phosphate carrier; SFC, succinate–fumarate carrier.

rat liver mitochondria (Chappell and Haarhoff, 1967) where it catalyzes a very active electroneutral exchange across the membrane of dicarboxylates (*e.g.*, malate and succinate) for inorganic phosphate (Chappell and Haarhoff, 1967; Palmieri *et al.*, 1970, 1971) and certain sulfur-containing compounds (*e.g.*, sulfate and thiosulfate) (Crompton, 1974a,b). It is inhibited by some impermeable dicarboxylate analogs (*e.g.*, *n*-butylmalonate) (Chappell *et al.*, 1968; Palmieri *et al.*, 1971) as well as by certain sulfydryl reagents (but not *N*-ethylmaleimide) (Meijer *et al.*, 1970; Palmieri *et al.*, 1974). The transport and biochemical characteristics of the rat protein have been extensively studied (see Indiveri *et al.*, 1993), but because the protein is not abundant, for a long time neither its sequence nor the coding sequence was established.

We recently identified the product of the yeast gene YLR348c (encoded on chromosome XII, accession number GenBank U19028) as the yeast dicarboxylate carrier from its transport properties and kinetic characteristics after bacterial expression and functional reconstitution (Palmieri *et al.*, 1996; Kakhniashvili *et al.*, 1997). The substrate specificity and the inhibitor sensitivity of the recombinant yeast protein are the same as those determined previously for the dicarboxylate carrier in rat mitochondria (Palmieri *et al.*, 1971) and after its purification from rat (Indiveri *et al.*, 1989) and from yeast (Lancar-Benba *et al.*, 1996). The expressed and reconstituted yeast dicarboxylate carrier displays K_m values for phosphate (1.65 $\pm$ 0.19 mM) and malate (0.56 $\pm$ 0.09 mM) very similar to the values obtained with the rat-liver carrier in both mitochondria (*i.e.*, 1.5 and 0.26 mM, respectively) (Palmieri *et al.*, 1971) as well as after purification (*i.e.*, 1.41 and 0.49 mM, respectively) (Indiveri *et al.*, 1989) (Table II). The V_{max} value for the expressed yeast dicarboxylate carrier (5.6 $\pm$ 0.7 mmol/ min $\times$ *g* protein at 25°C) is higher than the value reported for the rat-liver dicarboxylate carrier in mitochondria (*i.e.*, 70 nmol/min $\times$ mg protein at 9°C) (Palmieri *et al.*, 1971) and it is virtually the same as the value reported for the purified and reconstituted rat-liver dicarboxylate carrier (Indiveri *et al.* 1989). Furthermore, the V_{max} is independent of the type of substrate, as previously observed for the dicarboxylate carrier in mitochondria (Palmieri *et al.*, 1971) and after purification (Indiveri *et al.*, 1989) (see Table II).

THE SEQUENCE OF THE RAT MITOCHONDRIAL DICARBOXYLATE TRANSPORTER CLONED VIA DISTANT HOMOLOGS IN YEAST AND *Caenorhabditis elegans*

More recently, using the sequence of the identified yeast dicarboxylate carrier and the almost complete

Table II. Kinetic Parameters of Purified and Reconstituted Mitochondrial Dicarboxylate Carriers

Source	K_m(mM)		V_{max}[a] (mmol/min × g protein)
	Malate	Phosphate	
Rattus norvegicus (native)[b]	0.49 ± 0.05	1.41 ± 0.35	6.0 ± 1.6
Saccharomyces cerevisiae (recombinant)[c]	0.56 ± 0.09	1.65 ± 0. 19	5.6 ± 0.7
Caenorhabditis elegans (recombinant)[d]	0.54 ± 0.10	1.52 ± 0.36	22.6 ± 2.8
Rattus norvegicus (recombinant)[d]	0.78 ± 0.14	1.77 ± 0.27	6.8 ± 0.9

[a] The V_{max} values were calculated considering the amount of protein added to the reconstitution mixture.
[b] From Indiveri *et al.* (1989).
[c] From Palmieri *et al.* (1996).
[d] From Fiermonte *et al*., (1998b).

sequence of the *Caenorhabditis elegans* genome, we adopted a new strategy to clone the mammalian homologs of the yeast dicarboxylate carrier (Fiermonte *et al.*, 1998b). Yeast sequences with known biochemical function were compared with those of their mammalian counterparts; this showed that the homologous pairs are too distant for the yeast sequences (protein or DNA) to provide a feasible basis for cloning the mammalian proteins. Therefore, the sequence of the *S. cerevisiae* dicarboxylate carrier has been compared with the proteins encoded in the available genomic sequences of *C. elegans* (currently, this genome encodes at least 37 members of the mitochondrial carrier family). Homologs of the yeast dicarboxylate carrier in *C. elegans* were identified by screening the nematode (The Sanger Centre, Hinxton, U. K.) and the NCBI databases with the sequence of the *S. cerevisiae* protein using the BLASTP program. The two most closely related proteins (namely, K11G12.5 and B0432.4) were identical to the yeast dicarboxylate carrier in 37 and 30% of their residues, respectively. Both proteins were expressed in *E. coli*, reconstituted into phospholipid vesicles, and the former was demonstrated to be the *C. elegans* dicarboxylate carrier from its transport characteristics (Fiermonte *et al.*, 1998b). The sequence of the *C. elegans* dicarboxylate carrier was then compared with protein sequences encoded in mammalian expressed sequence tags (EST) using the TBLASTN program. In this way, two partial murine (AA199557, AA041737) were found to encode a fragment of a related protein. Synthetic oligonucleotides, based on their sequences, were used to amplify DNA segments from murine and rat cDNAs. Using these segments, the full-length murine and rat cDNAs were obtained by PCR experiments. The rat cDNA sequence of 1946 nucleotides encodes a protein with a molecular mass of 31455Da. This protein, expressed in *E. coli* and reconstituted into phospholipid vesicles, was shown to have transport specificity and other characteristics that are very similar to those of the dicarboxylate carrier isolated from rat liver mitochondria. In liposomes reconstituted with the *C. elegans* protein, the K_m are 0.54 ± 0.10 and 1.52 ± 0.36 mM for malate and phosphate, respectively; for the rat protein, the corresponding values are 0.78 ± 0.14 and 1.77 ± 0.27. The V_{max} of the recombinant rat dicarboxylate carrier is very close to the values determined previously for the rat liver native protein and the recombinant yeast protein (see Table II; Indiveri *et al.*, 1989; Palmieri *et. al.*, 1996). As shown in Table II, the dicarboxylate carrier has almost the same affinity constants for its main substrates in all species investigated. This argues in favor of a conserved metabolic significance.

Very recently, we cloned and sequenced the cDNA and the gene for the human dicarboxylate carrier (Fiermonte *et al.*, 1999). Furthermore, this gene was mapped to chromosome 17q25.3 (Pannone *et al.*, 1998). It is notable that the dicarboxylate carrier transcript, about 2.1 kb, is longer than the cDNA sequence, indicating that it may have an extensive 5′ noncoding region. By Northern and Western blot analyses it was shown that the dicarboxylate carrier is present in high amounts in rat mitochondria from liver and kidney and at lower levels in mitochondria from heart and brain (Fiermonte *et al.*, 1998b, 1999).

THE *Saccharomyces cerevisiae* NUCLEAR GENE ENCODING THE MITOCHONDRIAL DICARBOXYLATE CARRIER IS ESSENTIAL FOR GROWTH ON ETHANOL OR ACETATE AS THE SOLE CARBON SOURCE

Although metabolite transport across the mitochondrial inner membrane has been widely investi-

gated over the past 30 years, knowledge of the role played by individual mitochondrial transporters in cell metabolism is still limited. In most cases, the metabolic significance of these carriers has been inferred from their transport properties and organ distribution. In intact mitochondria of both high eukaryotic and yeast cells, accumulation of dicarboxylates (*e.g.*, succinate and malate) in the mitochondrial matrix was found to be dependent on the ΔpH across the membrane (Palmieri *et al.*, 1970; Quagliariello and Palmieri, 1968, 1970; Perkins *et al.*, 1973). Since the dicarboxylate–phosphate exchange is electroneutral, the ΔpH dependence of dicarboxylate uptake into the mitochondria is due to the reuptake of phosphate (as P_i^-/H^+ symport or P_i^-/OH^- antiport via the phosphate carrier) whose distribution across the membrane is in direct equilibrium with the pH gradient (Palmieri *et al.*, 1970). Subsequent studies in intact cells confirmed the accumulation of phosphate and dicarboxylates in the mitochondria with respect to the cytosol (Siess *et al.*, 1982). Thus, the first and obvious function of the dicarboxylate carrier is to catalyze the entry of Krebs cycle intermediates into the mitochondrion. Based on the observations that butylmalonate inhibits glucose production from pyruvate or lactate both in perfused rat liver (Williąmson *et al.*, 1970) and in isolated rat liver cells (Soling *et al.*, 1973), and that fluoromalate also inhibits gluconeogenesis (Berry and Kuan, 1972), it is generally accepted that the dicarboxylate carrier plays an important role in gluconeogenesis by exporting malate out of the mitochondria in exchange for cytosolic phosphate (for a review see Meijer and van Dam, 1974). In addition, in *S. cerevisiae*, the dicarboxylate carrier was assumed to be involved in gluconeogenesis from pyruvate, lactate, and glycerol (Wills *et al.*, 1984), as well as from ethanol and acetate (Wills *et al.*, 1986). The very low activity of the dicarboxylate carrier in heart was also interpreted in favor of the dicarboxylate carrier role in gluconeogenesis (Sluse *et al.*, 1971) because of the lack of the gluconeogenic function in this tissue. However, net outflow of malate would require the operation of the phosphate carrier against its driving force (the ΔpH) to recycle back to the cytosol the phosphate exchanged for malate across the mitochondrial membrane. Furthermore, it was subsequently shown that butylmalonate and *p*-iodobenzylmalonate, another strong inhibitor of the dicarboxylate carrier, inhibit gluconeogenesis from externally added malate to a similar extent to pyruvate and lactate (Rognstad and Katz, 1973), indicating that butylmalonate in the intact cell has an inhibitory effect elsewhere in the gluconeogenic pathway.

To gain insight into the physiological role of the dicarboxylate carrier, we deleted the nuclear gene encoding this carrier in *S. cerevisiae* by homologous recombination of the HIS3 gene (Palmieri *et al.*, 1999). The deletion mutant (ΔDIC strain) failed to grow on ethanol or acetate as the sole carbon source, but was viable on other nonfermentative (pyruvate, lactate, glycerol, and oxaloacetate) and fermentative (glucose and galactose) carbon sources. Furthermore, the growth of the dicarboxylate carrier knockout strain on ethanol or acetate was restored by the addition of low concentrations of oxaloacetate, aspartate, glutamate, oxoglutarate, and citrate, but not of succinate, leucine, and lysine. ΔDIC mitochondria were found to be impaired in malate–phosphate and succinate–phosphate exchanges, which are characteristic reactions of the mitochondrial dicarboxylate carrier. However, cellular respiration, the expression of other mitochondrial carriers, and the stability and coupling of the mitochondria were all unaffected by the absence of the dicarboxylate carrier activity. Furthermore, mitochondria isolated from the deletion strain transformed with a multicopy plasmid carrying the dicarboxylate carrier ORF catalyzed a very active uptake of malate in exchange for phosphate: this activity was markedly inhibited by butylmalonate by SH-blocking reagents, and by known dicarboxylate carrier substrates (Palmieri *et al.*, 1999). In the yeast *S. cerevisiae* succinate can enter the mitochondria through two transport systems: the dicarboxylate carrier (Palmieri *et al.*, 1996) and the succinate–fumarate carrier (Palmieri *et al.*, 1997b). However, although the succinate–fumarate carrier requires fumarate as countersubstrate, the dicarboxylate carrier catalyzes the import of succinate in exchange for internal phosphate. Since the latter is recycled into mitochondria by the phosphate carrier, the combined activity of the dicarboxylate carrier and the phosphate carrier leads to a net uptake of succinate. In wild-type *S. cerevisiae*, succinate, produced in the cytosol by the glyoxylate pathway and imported by the dicarboxylate carrier, is converted, within the mitochondria, to fumarate and oxaloacetate. The conversion of succinate to Krebs cycle intermediates allows the oxidation of acetyl-CoA produced from ethanol or acetate and triggers the activity of the succinate–fumarate carrier (see Fig. 2). The anaplerotic role of the dicarboxylate carrier is in agreement with the lack of growth of the *S. cerevisiae* ΔDIC strain on ethanol or acetate and is supported by the effect of oxaloacetate and other compounds, which can generate Krebs cycle

intermediates, in restoring the ΔDIC strain viability on ethanol or acetate. Taken together, our results (Palmieri et al., 1996, 1997b, 1999) provide evidence for the anaplerotic role of the dicarboxylate carrier in cell metabolism by catalyzing the entry of Krebs cycle intermediates into the mitochondria and question the proposal that this carrier is involved in gluconeogenesis from ethanol or acetate by exporting malate from the mitochondria (Wills *et al.*, 1986). An argument against the export of malate is the ΔpH dependence of malate movement across the mitochondrial membrane (Palmieri *et al.*, 1970). Furthermore, a dicarboxylate–dicarboxylate exchange via the dicarboxylate carrier is very unlikely due to the high intramitochondrial phosphate concentration (Siess *et al.*, 1982). Finally, after the recent discovery of the succinate–fumarate carrier in yeast (Palmieri *et al.*, 1997b) the proposed role of the dicarboxylate carrier for gluconeogenesis is unnecessary. In the presence of ethanol or acetate, when sufficient tricarboxylate cycle intermediates are provided by the dicarboxylate carrier, it is useful for *S. cerevisiae* cells to direct the carbon flux mainly to synthetic pathways. This idea is supported by one of our findings, *i.e.*, the repression of the dicarboxylate carrier expression by ethanol or acetate (Palmieri *et al.*, 1999). Clearly a lower activity of the dicarboxylate carrier would favor the utilization of cytosolic succinate by the succinate–fumarate carrier and, thereby, the gluconeogenic pathway. The presence of the dicarboxylate carrier in heart and brain (Fiermonte *et al.*, 1998b, 1999), where gluconeogenesis does not operate, suggests that this carrier functions in these tissues, as in yeast, exclusively catalyzing supply of substrates to the Krebs cycle.

IDENTIFICATION OF THE YEAST ARG11 GENE PRODUCT AS THE MITOCHONDRIAL ORNITHINE CARRIER PROTEIN

The biosynthesis of arginine in *S. cerevisiae* involves five enzymes in the matrix of mitochondria, that produce ornithine from imported glutamate, and three enzymes in the cytosol, which convert ornithine exported from the mitochondria into arginine (Davis, 1986). Yeast cells with mutations in the ARG11 gene grow poorly in the absence of arginine (Crabeel *et al.*, 1996), and in one mutant, MG409 (Arg 11-1), the glutamate pool was increased and the arginine and ornithine pools were decreased (Delforge *et al.*, 1975). Therefore, it was proposed that the protein encoded by ARG11 provides one link between the intramitochondrial and cytosolic parts of the arginine biosynthetic pathway, either by importing glutamate into the mitochondrion, or by exporting ornithine from the organelle into the cytosol (Crabeel *et al.*, 1996). The ARG11 gene (or YOR130c), located on chromosome XV, encodes a protein with a sequence containing the characteristic features of the mitochondrial carrier family. According to the strategy employed in our laboratories, the yeast ARG11 protein was overproduced in *Escherichia coli*, solubilized in the presence of sarkosyl, and reconstituted into liposomes. Studies of its transport characteristics showed it to be an ornithine carrier, since it catalyzed ornithine–ornithine and ornithine–H^+ exchanges (Palmieri *et al.*, 1997a). The homo-exchange is much faster than the ornithine–H^+ exchange, and, in this respect, the yeast ornithine carrier is similar to the mitochondrial phosphate and carnitine carriers (Stappen and Kramer, 1993; Indiveri *et al.*, 1991). The reconstituted ARG11 protein transported arginine and lysine less efficiently than ornithine, but citrulline and histidine were not transported. Therefore, the substrate specificity of the ARG11 protein is confined to a narrow range of basic amino acids, and is similar to that of the rat ornithine carrier (Indiveri *et al.*, 1992, 1994), except that citrulline is not transported by the yeast protein. Furthermore, ornithine transport catalyzed by the recombinant and reconstituted ARG11 protein was inhibited by sulfydryl reagents (both organic mercurials and maleimides) and various cations, which also inhibit the rat ornithine carrier (Indiveri *et al.*, 1992, 1994). The K_m and V_{max} values for ornithine–ornithine exchange at 25°C were 0.11 ± 0.01 mM and 1.1 ± 0.3 mmol/min pergram protein, respectively. The K_m for ornithine uptake is close to that of the rat ornithine carrier (Indiveri *et al.*, 1994), but the V_{max} is lower than that of the purified rat protein (3.2 ± 0.7 mmol/min pergram protein) (Indiveri *et al.*, 1994). The other two substrates, arginine and lysine, competitively inhibited ornithine uptake. Their K_i values were 0.6 ± 0.1 and 1.3 ± 0.2 mM, respectively. The K_i value for lysine is close to that of the rat protein (Indiveri *et al.*, 1994). In other experiments, we found that, first, the uptake of ornithine by unloaded proteoliposomes (*i.e.*, in the absence of countersubstrate) increased markedly upon decreasing the internal pH from 8.0 to 6.0 (at fixed external pH); second, the addition of pH 6.0 buffer to ornithine-loaded proteoliposomes with an internal pH of 8.0 induced greater efflux of ornithine than an external pH of 8.0; and third,

both influx and efflux of ornithine were prevented by inhibitors of the ornithine–ornithine exchange (Palmieri *et al.*, 1997a). Therefore, the reconstituted flux of ornithine across the proteoliposomal membrane is dependent on the transmembrane ΔpH, indicating that the ARG11 protein, *i.e.*, the yeast ornithine carrier, can catalyze the transport of ornithine in exchange for H^+. On the basis of these findings, we proposed that the main physiological role of the yeast ornithine carrier is to transport ornithine, synthesized from glutamate in the mitochondrial matrix, to the cytosol where it is converted to arginine and polyamines (see Fig. 3). This export of ornithine occurs at the expense of the proton motive force generated across the mitochondrial membrane by electron transport. At high cytosolic concentrations of arginine, the ornithine carrier can catalyze the entry of arginine or lysine into mitochondria in exchange for ornithine. In the mitochondrial matrix, arginine inhibits acetylglutamate synthase and acetylglutamate kinase, the first two enzymes of its biosynthetic pathway in a feedback mechanism (Fig. 3) and both arginine and lysine are consumed in protein synthesis. Recent work on mitochondria, isolated from an ARG11 knockout strain, has confirmed our findings (Soetens *et al.*, 1998). Interestingly, transport of ornithine and arginine was strongly reduced, but not completely abolished, in agreement with the leaky phenotype of the ARG11 knockout mutants. The inner membranes of rat liver mitochondria were suggested to have two transport systems for ornithine, one for ornithine (and lysine) via an ornithine–H^+ antiport (McGivan *et al.*, 1977; Hommes *et al.*, 1983), and the second for ornithine import in exchange for citrulline (Bradford and McGivan, 1980). The ornithine–citrulline carrier (purified, but not sequenced, from rat liver mitochondria) (Indiveri *et al.*, 1992) transports ornithine, lysine, arginine, and citrulline by an electroneutral antiport mechanism (Indiveri *et al.*, 1992, 1997). In addition to the ornithine–citrulline exchange, the purified rat protein catalyzes an ornithine–H^+ exchange, although with a lower efficiency (Indiveri *et al.*, 1999). Therefore, the mammalian carrier seems to have evolved to accept citrulline as substrate, and fulfill the important function of exchanging cytosolic ornithine and intramitochondrial citrulline—an essential step in the urea cycle.

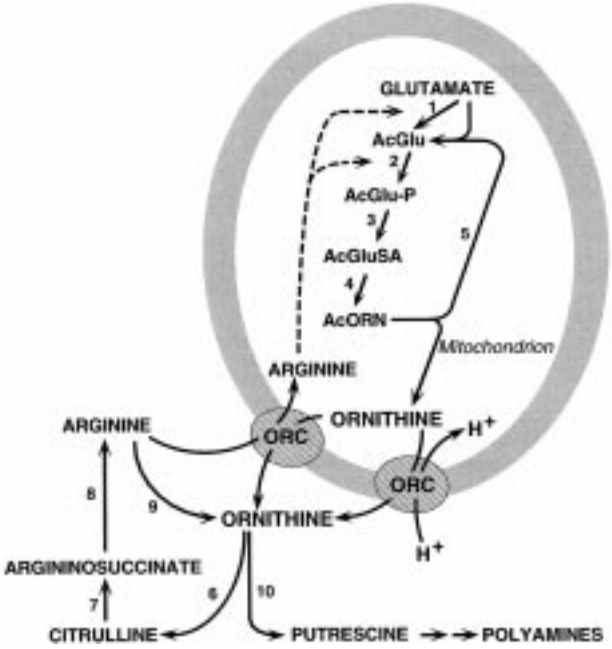

Fig. 3. Compartmentalization of selected enzymes involved in arginine and polyamine biosynthesis and the role of the mitochondrial ornithine carrier. Two molecules of ornithine carrier (ORC) are depicted in the mitochondrial membrane in order to show two independent physiologically important transport modes catalyzed by the carrier. The broken lines indicate feedback inhibition by arginine of acetylglutamate synthase and acetylglutamate kinase. ORC, ornithine carrier; AcGlu, *N*-acetylglutamate; AcGlu-P, *N*-acetyl-γ-glutamyl-phosphate; AcGluSA, *N*-acetyl-γ-glutamate semialdehyde; AcORN, $^{\alpha}$*N*-acetylornithine. The following enzymes are involved; (1) acetylglutamate synthase; (2) acetylglutamate kinase; (3) acetylglutamyl-phosphate reductase; (4) acetylornithine transaminase; (5) acetylornithine glutamate acetyltransferase; (6) ornithine carbamoyltransferase; (7) argininosuccinate synthetase; (8) argininosuccinate lyase; (9) arginase; (10) ornithine decarboxylase.

CONCLUSIONS

As described in this short review, overexpression of putative mitochondrial transporter genes identified from analysis of the yeast genome, followed by purification and functional reconstitution of the gene products, has provided information on the primary structure of three mitochondrial carriers, which had not been previously sequenced in any organism. These results have enabled the dicarboxylate, the ornithine, and the succinate–fumarate carriers to be assigned to the mitochondrial carrier protein family based on both structural and functional considerations. The strategy used in the studies reviewed here should make it possible to identify the biochemical function of other yeast genes encoding members of the mitochondrial carrier family, and this identification, together with the characterization of the respective knockout strains, should bring new insight into the physiological significance

of the individual mitochondrial carrier proteins in cell metabolism. Furthermore, as exemplified here for the dicarboxylate carrier, it may be possible to identify the mammalian (human) mitochondrial carriers via the distant homologs in *S. cerevisiae* and *C. elegans*. In addition, high-yield expression of mitochondrial transporters allows the use of site-directed mutagenesis for elucidating structure–function relationships, and may provide sufficient amounts of active transport proteins for crystallization.

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