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Identification of the mitochondrial carnitine carrier in *Saccharomyces cerevisiae*

Luigi Palmieri[a], Francesco M. Lasorsa[a], Vito Iacobazzi[a], Michael J. Runswick[b], Ferdinando Palmieri[a], John E. Walker[b,*]

[a] *Department of Pharmaco-Biology, Laboratory of Biochemistry and Molecular Biology, University of Bari, Via Orabona 4, 70125 Bari, Italy*
[b] *The M.R.C. Dunn Human Nutrition Unit, Hills Road, Cambridge CB2 2DH, UK*

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Abstract The mitochondrial carrier protein for carnitine has been identified in *Saccharomyces cerevisiae*. It is encoded by the gene CRC1 and is a member of the family of mitochondrial transport proteins. The protein has been over-expressed with a C-terminal His-tag in *S. cerevisiae* and isolated from mitochondria by nickel affinity chromatography. The purified protein has been reconstituted into proteoliposomes and its transport characteristics established. It transports carnitine, acetylcarnitine, propionylcarnitine and to a much lower extent medium- and long-chain acylcarnitines.

Key words: Yeast; Mitochondrion; Transport; CRC1 gene; Carnitine carrier; YOR100c

1. Introduction

Mitochondria are impermeable to acyl-CoAs of any chain length. To traverse the inner mitochondrial membrane, acyl residues are transiently transferred to L-carnitine by specific carnitine acyltransferases. The carnitine/acylcarnitine translocase (CACT) from mammalian mitochondria catalyses the entry of acylcarnitines of various lengths in exchange for free carnitine into the mitochondrial matrix where the acyl moieties are oxidised by the enzymes of the β-oxidation pathway and the tricarboxylic acid cycle [1,2]. Because of its fundamental role in bioenergetics, the basic functional properties of CACT have been extensively investigated in intact mitochondria and with purified protein incorporated into liposomes [3]. Recently, CACT has been cloned from man and rat [3,4] and its primary structure shows that it is a member of the mitochondrial carrier family [5–7]. Human CACT deficiency impairs fatty acid oxidation [4] and CACT was the first gene for a mitochondrial carrier associated with a human disease [4].

Fungal genes encoding CACT have not been identified hitherto nor has its product been isolated and characterised. However, several facts point to its existence in *Saccharomyces cerevisiae*. For example acetylcarnitine is known to be involved in shuttling acetyl groups across the inner mitochondrial membrane [8], and two carnitine acetyltransferases are known [9]. One, YAT1, is located at the outer membrane of mitochondria and is ethanol-inducible. The other, encoded by the CAT2 gene and found in both mitochondria and peroxisomes, accounts for >95% of the total carnitine acetyltransferase activity in yeast [10]. *S. cerevisiae* encodes 35 members of the mitochondrial transporter family [11]. The transport functions of most family members are unknown. Among them, the CRC1[1] gene product is the most closely related to CACT (29% identity). This gene is the only one in *S. cerevisiae* encoding a member of the mitochondrial carrier family that has a promoter region containing an ORE (oleate-responsive element) and its transcription has been shown to be induced by oleate [12].

In the present work, the CRC protein has been over-expressed in *S. cerevisiae* with a C-terminal histidine tail and purified by affinity chromatography. Upon functional reconstitution into liposomes, it has been shown to be a carnitine carrier that also transports acetylcarnitine, propionylcarnitine and, much less efficiently, medium- and long-chain acylcarnitines. This report is the first information about the molecular properties of the yeast carnitine carrier, and it provides the definitive identification of its gene.

2. Materials and methods

2.1. Construction of the CRC expression plasmid

The coding sequence for CRC (YOR100c; GenBank accession number AJ250124) was amplified from *S. cerevisiae* genomic DNA via PCR. Forward and reverse oligonucleotide primers were synthesised corresponding to the extremities of the CRC1 sequence with additional *Hin*dIII and *Bam*HI sites, respectively. The reverse primer also contained 18 extra bases encoding a 6-histidine tag immediately before the stop codon. The PCR product was cloned into the expression vector pYES2 (Invitrogen). The resulting expression plasmid was introduced in the *S. cerevisiae* YPH499 strain [13] (wild-type), and transformants (CRC1-pYES2 cells) were selected for uracil auxotrophy. Other conditions have been given before [14–16].

2.2. High-level expression and purification of the CRC protein

Yeasts were precultured in synthetic medium as previously described [16]. For the preparation of mitochondria yeast cells were grown at 30°C to mid-log phase in YP medium (1% yeast extract, 2% Bacto-peptone, pH adjusted to 4.8 with HCl) supplemented with 3% glycerol and 0.1% glucose. Galactose (0.5%) was added 6 h before harvesting (5 min at 3000×*g*). Mitochondria were isolated according to standard procedures [17] and solubilised in buffer A (500 mM NaCl, 10 mM PIPES, pH 7.0) containing 2% Triton X-100 (w/v) and 4 mg/ml cardiolipin, at a final concentration of 0.4 mg protein/ml. After incubation for 20 min at 4°C, the mixture was spun at 138 000×*g* for 20 min. The supernatant extract (1.1 ml) was incubated batchwise for 40 min with 0.35 ml Ni-NTA agarose (Qiagen) previ-

*Corresponding author. Fax: (44)-1223-252705.
E-mail: walker@mrc-dunn.cam.ac.uk

[1] The name CRC1 has been reserved for the gene encoding the yeast carnitine carrier.

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ously equilibrated with buffer A. Then the resin was packed into a column (0.5 cm internal diameter) and washed with 10 ml of buffer B (100 mM NaCl, 0.4% Triton X-100, 10 mM PIPES, pH 7.0) in the presence of 10 mM imidazole. Elution was carried out using an imidazole gradient (10–100 mM imidazole in buffer B). Pure CRC protein eluted at an imidazole concentration of 80 mM. Protein was determined by the Lowry method modified for the presence of detergent [18] or by laser densitometry [11,15].

2.3. Protein chemical characterisation of over-expressed CRC

The analysis of proteins by SDS-PAGE and N-terminal sequencing has been described previously [14]. For internal sequence determinations, the purified CRC protein was digested with cyanogen bromide as described [3]; the peptides were separated by SDS-PAGE as described by Schägger and von Jagow [19], transferred to poly(vinylidene difluoride) membranes, stained with Coomassie blue dye, and their N-terminal sequences determined with a pulsed liquid protein sequencer (Applied Biosystems 477A).

2.4. Activity assays

CRC was reconstituted into proteoliposomes as described for other mitochondrial carriers [15,20]. External substrate was removed from proteoliposomes on a Sephadex G-75 column preequilibrated with buffer C (50 mM NaCl, 10 mM PIPES, pH 7.0). Transport at 25°C was started by adding [^{3}H]carnitine (from Amersham Pharmacia Biotech) to the proteoliposomes and terminated by addition of 40 mM pyridoxal 5′-phosphate and 15 mM bathophenanthroline (the 'inhibitor-stop' method [20]). In control samples, the inhibitors were added with the labelled substrate. The transport activities were calculated from the experimental values minus the controls. For kinetic measurements, transport was stopped after 1 min, i.e. within the initial linear range of [^{3}H]carnitine uptake into the proteoliposomes. Various other transport activities were also assayed by the inhibitor-stop method. For efflux measurements, the internal substrate pool of the proteoliposomes was made radioactive by carrier-mediated exchange equilibration [20] with 3 mM [^{3}H]carnitine added at high specific radioactivity. After 60 min, the residual external radioactivity was removed by passing the proteoliposomes again through a column of Sephadex G-75. Efflux was started by adding unlabelled external substrate or buffer C alone and terminated by adding the inhibitors indicated above.

3. Results and discussion

3.1. Over-expression of CRC

CRC was expressed at high level in yeast mitochondria from the multicopy vector pYES2 (Fig. 1, lane 2). The protein has an apparent molecular mass of about 40 kDa (the calculated molecular mass including the initiator methionine and the histidine tag is 35 550 Da). It was estimated to represent about 12% of the total mitochondrial protein. The presence of the C-terminal histidine tail allowed the CRC to be purified by Ni^+-agarose chromatography (Fig. 1, lane 3). About 0.2 mg of purified protein was obtained per litre of yeast culture. The purified protein does not have a free α-amino group, and so its identity was confirmed by sequencing two internal cyanogen bromide fragments. Three fragments were detected on SDS-PAGE gels. Like the intact protein the upper band (approximately 16 kDa) gave no N-terminal sequence, whereas the two lower bands (12 kDa and 5 kDa) gave sequences identical to residues 145–154 and 277–286 of the CRC (AAAGFISAIP and LSATKEIYLQ, respectively).

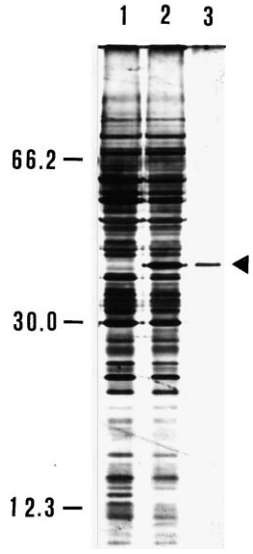

Fig. 1. Purification of the over-expressed yeast carnitine carrier. Proteins were separated by SDS-PAGE and stained with silver nitrate. The positions of molecular weight markers are shown on the left in kDa. Lanes 1 and 2, mitochondrial protein (10 μg) from wild-type (lane 1) and CRC1-pYES2 strain (lane 2). Cells were harvested 6 h after addition of galactose. Lane 3, CRC protein (1.5 μg) purified from mitochondria in lane 2.

Table 1
Carnitine transport by yeast CRC and the effects of inhibitors

Protein	Inhibitor	[^{3}H]Carnitine uptake (μmol/15 min/g protein)
YPH499 extract	none	39
CRC1-pYES2 extract	none	825
Pure CRC	none	7940
Pure CRC	mersalyl	238
Pure CRC	*p*-chloromercuribenzensulphonate	476
Pure CRC	mercuric chloride	318
Pure CRC	*N*-ethylmaleimide	2461
Pure CRC	pyridoxal 5′-phosphate	1746
Pure CRC	bathophenanthroline	2461
Pure CRC	α-cyanocinnamate	7146
Pure CRC	carboxyatractyloside	7543
Pure CRC	phenylsuccinate	8845
Pure CRC	butylmalonate	8099
Pure CRC	1,2,3-benzenetricarboxylate	8560

The [^{3}H]carnitine/carnitine exchange was measured in liposomes reconstituted with mitochondrial yeast extracts of wild-type or CRC1-pYES2 cells, and with the CRC protein purified from the latter strain. The proteoliposomes were preloaded with 13 mM carnitine and transport was started by adding 0.1 mM [^{3}H]carnitine. Thiol reagents, pyridoxal 5′-phosphate, carboxyatractyloside and α-cyanocinnamate were added 3 min before the labelled substrate; other inhibitors were added together with [^{3}H]carnitine. The final concentration of all inhibitors was 15 mM, except for organic mercurials and carboxyatractyloside (0.1 mM), α-cyanocinnamate and *N*-ethylmaleimide (2 mM). Similar results were obtained in 2–4 independent experiments.

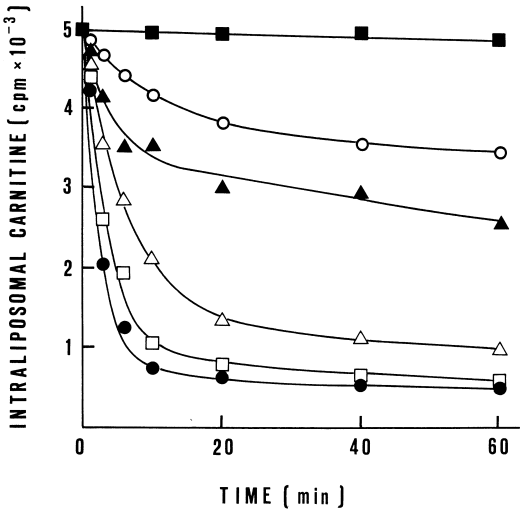

Fig. 2. Efflux of [^{3}H]carnitine from proteoliposomes. Proteoliposomes were reconstituted in the presence of 3 mM carnitine, and then the internal substrate pool was labelled by carrier-mediated exchange equilibration. After removal of external substrate by Sephadex G-75 chromatography, the efflux of [^{3}H]carnitine was started by adding buffer C alone (uniport, ○) or 0.2 mM L-carnitine (□), 0.2 mM L-acetylcarnitine (●), 0.2 mM L-nonanoylcarnitine (△) or 0.2 mM L-palmitoylcarnitine (▲) or 40 mM pyridoxal 5′-phosphate and 15 mM bathophenanthroline (■) in the same buffer.

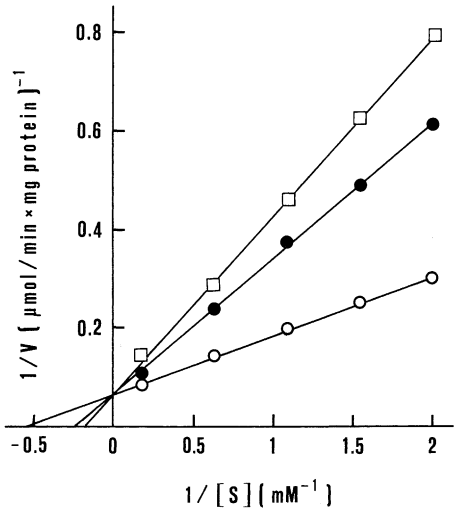

Fig. 3. Effect of external carnitine concentration on the rate of carnitine/carnitine exchange in reconstituted liposomes and competitive inhibition by acetylcarnitine and propionylcarnitine. [^{3}H]Carnitine was added at various concentrations to proteoliposomes containing 20 mM carnitine. Except in controls (○), 2.5 mM L-acetylcarnitine (□) or 2.5 mM L-propionylcarnitine (●) was added simultaneously with the labelled substrate.

The yeast CRC catalysed transport of carnitine (see Table 1). The uptake of [^{3}H]carnitine was measured in proteoliposomes that had been reconstituted with a Triton X-100 extract of CRC1-pYES2 mitochondria and that contained 13 mM carnitine. The uptake was more than 20-fold higher than by similar proteoliposomes that had been reconstituted with a mitochondrial extract from wild-type cells. Furthermore, the purified recombinant CRC reconstituted into proteoliposomes had an even greater carnitine carrier activity. The same proteoliposomes did not catalyse homoexchanges of phosphate, ATP, citrate, malate, oxoglutarate, succinate, fumarate, glutamate, aspartate, glutamine, and ornithine (external concentration, 1 mM; internal concentration, 10 mM) (data not shown), and no carnitine/carnitine homoexchange was detected with purified CRC that had been boiled before incorporation into liposomes (not shown).

The activity of the purified CRC protein was markedly inhibited by sulphydryl reagents, pyridoxal 5′-phosphate and bathophenanthroline (Table 1). In contrast, inhibitors of other mitochondrial carriers such as α-cyanocinnamate, carboxyatractyloside, phenylsuccinate, butylmalonate and 1,2,3-benzenetricarboxylate had very little or no effect on the reconstituted carnitine transport. These results show that the inhibition characteristics of the recombinant CRC protein are similar to those previously described for the mammalian carnitine carrier [1,21].

3.2. Substrate specificity

The substrate specificity of the purified CRC was investigated in greater detail by measuring the uptake of external [^{3}H]carnitine into proteoliposomes which had been pre-loaded with different compounds. As shown in Table 2, [^{3}H]carnitine was efficiently taken up by proteoliposomes containing 13 mM internal carnitine, acetylcarnitine or propionylcarnitine. A much lower activity was observed in the presence of internal D-stereoisomers. Because of their detergent properties, the effect of intraliposomal medium and long-chain fatty acid esters of carnitine was tested at a concentration of 2 and 1 mM, instead of 13 mM. In separate experiments neither L-nonanoylcarnitine nor L-palmitoylcarnitine up to 2 mM had any appreciable solubilising effect on reconstituted liposomes [21]. The exchange of [^{3}H]carnitine for internal octanoylcarnitine, nonanoylcarnitine, lauroylcarnitine, myristoylcarnitine and palmitoylcarnitine was much lower than for

Table 2
Effect of internal substrate on the transport properties of proteoliposomes containing recombinant yeast CRC

Internal substrate	Concentration (mM)	Carnitine transport (µmol/15 min/g protein)
None	–	83
L-Carnitine	13	8890
D-Carnitine	13	3349
DL-Carnitine	13	4791
L-Acetylcarnitine	13	6446
DL-Acetylcarnitine	13	5154
L-Propionylcarnitine	13	6090
L-Carnitine	2	3590
L-Acetylcarnitine	2	3458
L-Propionylcarnitine	2	3383
L-Nonanoylcarnitine	2	1312
L-Palmitoylcarnitine	2	391
L-Carnitine	1	2203
L-Acetylcarnitine	1	2853
L-Propionylcarnitine	1	2467
DL-Octanoylcarnitine	1	419
L-Nonanoylcarnitine	1	412
DL-Lauroylcarnitine	1	695
DL-Myristoylcarnitine	1	245
L-Palmitoylcarnitine	1	181
DL-Stearoylcarnitine	1	121

Proteoliposomes were pre-loaded internally with various substrates at the indicated concentrations. Transport was started by the external addition of 0.1 mM [^{3}H]carnitine, and stopped after 15 min. Similar results were obtained in three independent experiments.

carnitine, acetylcarnitine and propionylcarnitine (see Table 2). Therefore medium- and long-chain fatty acid esters of carnitine are poor substrates for the *S. cerevisiae* carnitine carrier. In this respect, the yeast CRC differs from the rat homologue [1,21,22]. No exchange was observed with internal 13 mM arginine, glutamine, glutamate, phosphate, oxoglutarate, succinate, fumarate, pyruvate, ornithine, betaine, choline, citrate and ATP (not shown). The residual activity in the presence of these substrates was virtually the same as the activity observed in their absence.

The rat carnitine carrier catalyses a low unidirectional transport (uniport) of carnitine in addition to the carnitine/acylcarnitine exchange [23]. In order to obtain further information about the transport characteristics of CRC, the efflux of [^{3}H]carnitine from pre-labelled active proteoliposomes was studied as it provides a more convenient assay for unidirectional transport [20]. In Fig. 2 the time course of the uniport measured as efflux of 3 mM [^{3}H]carnitine is compared to the exchange reaction measured under the same conditions in the presence of various substrates. In the absence of external substrate, a slow efflux of [^{3}H]carnitine was detected; its rate (112 cpm/min) was much lower than that observed in the presence of 0.2 mM acetylcarnitine (1612 cpm/min). Medium- and long-chain fatty acids esters of carnitine (i.e. L-nonanoylcarnitine and L-palmitoylcarnitine) were also exchanged for [^{3}H]carnitine, although at a lower rate than external carnitine and acetylcarnitine.

3.3. Kinetic characteristics of reconstituted CRC

The basic kinetic data of the recombinant purified CRC were determined by measuring the initial transport rate at various external carnitine concentrations, in the presence of a constant saturating internal concentration of 20 mM carnitine. The K_m and V_{max} values for carnitine exchange at 25°C, from a typical experiment (see Fig. 3), were 1.79 mM and 15.1 mmol/min per g protein, respectively, and the average values from ten experiments were 1.9 ± 0.2 mM and 15.1 ± 3.7 mmol/min per g protein, respectively. Acetylcarnitine and propionylcarnitine, which are also good substrates of CRC, inhibited carnitine uptake competitively. Their K_i values for [^{3}H]carnitine uptake were 1.5 ± 0.2 mM and 2.0 ± 0.1 mM, respectively (average of four experiments). In similar experiments, the inhibition constants of medium- and long-chain acylcarnitines for the rat CRC homologue were found to be in the micromolar range [21,22] In contrast, L-nonanoylcarnitine and L-palmitoylcarnitine at a concentration of 0.2 mM (i.e. about 30-fold higher than their K_i for the rat carnitine carrier) did not significantly inhibit the rate of [^{3}H]carnitine/carnitine exchange catalysed by the CRC protein (not shown).

3.4. Conclusions

The transport characteristics of the yeast CRC protein show that it is a carnitine transporter. It also transports short-chain acylcarnitines with high efficiency and medium- and long-chain derivatives to a much lower extent. In contrast to mammals, where only very long-chain fatty acids are shortened in peroxisomes and all the other acyl groups are transported and oxidised in the mitochondria, in *S. cerevisiae* fatty acid β-oxidation is restricted to peroxisomes [24]. In the light of the transport characteristics of the yeast CRC protein it is likely that the main physiological function of the yeast carnitine carrier is to import acetylcarnitine (generated in the peroxisomes by CAT2 protein) into the mitochondrion in exchange for free carnitine. This conclusion is consistent with the co-regulation by oleate of the CRC1 gene and the CAT2 gene encoding the major carnitine acetyltransferase [10,12]. In mammals the carnitine carrier has probably evolved to bind and transport medium- and long-chain acylcarnitines with high efficiency.

In addition to being involved in the complete oxidation of fatty acids, the CRC protein may also participate in the oxidation of the fermentation product ethanol, which is converted to acetate and then activated to acetyl-CoA in the cytosol. This suggestion implies transfer of the acetyl moiety to L-carnitine by the YAT1 protein located in the mitochondrial outer compartment. Expression of this carnitine acetyltransferase is induced by ethanol and acetate, but not oleate [9,12]. It is noteworthy that a closely related homologue in *Aspergillus nidulans* (named ACUH) is required for the mycelium growth on long-chain fatty acids or acetate [25]. In addition, propionylcarnitine transport into the mitochondrion by CRC may be needed for propionate metabolism which, in *S. cerevisiae*, occurs via the methylcitrate pathway [26] within the mitochondrion (J.T. Pronk Delft, personal communication). Like its mammalian homologue, the most likely sub-cellular localisation of the CRC protein is the mitochondrial inner membrane. It has a N-terminal amphipathic stretch (absent from mammalian counterparts), which has some of the characteristic features of presequences that direct proteins to mitochondria [25]. It has been suggested that the rat carnitine carrier may also be targeted to peroxisomes [27]. Further experimentation is required to examine whether CRC is also present in the peroxisomal membranes.

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