Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1758 (2006) 124–131
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Functional reconstitution into liposomes and characterization of the carnitine transporter from rat liver microsomes

Annamaria Tonazzi [a], Michele Galluccio [b], Francesca Oppedisano [b], Cesare Indiveri [b,*]

[a] National Research Council (CNR) Institute of Biomembranes and Bioenergetics (IBBE), via Amendola 165/A 70126 Bari, Italy
[b] Department of Cellular Biology, University of Calabria, Via P.Bucci 4c 87036 Arcavacata di Rende, Italy

Received 30 November 2005; received in revised form 8 January 2006; accepted 9 January 2006
Available online 30 January 2006

Abstract

The carnitine transporter was solubilized from rat liver microsomes with Triton X-100 and reconstituted into liposomes, after addition of Triton X-114, by removing the detergent from mixed micelles by hydrophobic chromatography on Amberlite (Bio-Beads SM 2). The reconstitution was optimized with respect to the detergent/phospholipid ratio, the protein concentration, and the number of passages through a single Amberlite column. The reconstituted carnitine transporter catalyzed a first-order uniport reaction inhibited by $HgCl_2$ and DIDS. The IC_{50} for $HgCl_2$ was 0.16±0.03 mM. The reconstituted transporter also catalyzed carnitine efflux from the proteoliposomes; the efflux was stimulated by externally added long-chain acylcarnitines. Besides carnitine, ornithine, arginine, glutamine and lysine were taken up by the reconstituted liposomes with lower efficiency respect to carnitine. Optimal activity was found at pH 8.0. The Km for carnitine on the external side of the transporter was 10.9±0.16 mM. The activation energy of the carnitine transport derived by Arrhenius plot was 16.1 kJ/mol.

Keywords: Endoplasmic reticulum; Microsome; Membrane; Transport; Liposome; Carnitine; Reconstitution

1. Introduction

Cytosolic acyl-CoA containing acyl-groups of different lengths (from acetyl to long chain fatty acyl), are substrates for several metabolic pathways in at least three different organelles, i.e., mitochondria, peroxisomes and endoplasmic reticulum. Since CoA pools are sequestered in the different cellular compartments, the acyl-CoA is virtually transported into the various sub-cellular locations by shuttle systems that perform the translocation of the acyl unit as acyl-carnitine [1–3]. The shuttle systems need both transferase enzymes for the transfer of the acyl-groups from the CoA to carnitine and vice versa, and transport systems to enable the translocation of the carnitine derivatives. The most well known of these systems, the mitochondrial carnitine shuttle, consists of: (i) an extramitochondrial carnitine acyl-transferase associated to the outer mitochondrial membrane; (ii) a transmembrane carnitine/acylcarnitine translocase, which is an integral inner membrane protein, specific for carnitine and acylcarnitine that catalyzes the antiport of carnitine and acylcarnitine and (iii) an intramitochondrial carnitine acyl-transferase, associated with the inner membrane and protruding into the mitochondrial matrix [1–5]. A shuttle system must exist in peroxisomes where long chain acyl groups are shortened to subsequently be transferred to mitochondria for the complete oxidation; the presence of acyltransferase and transport activities in peroxisomes support the existence of this metabolic process [1,6–9]. Enzymes that require acyl-CoA thioesters in the endoplasmic reticulum include: an intralumenal oriented diacylglycerol acyl transferase that is involved in triacylglycerol synthesis for the VLDL production [10,11]; an internal acyl-CoA:cholesterol

Abbreviations: NEM, N-ethylmaleimide; NPheM, N-phenylmaleimide; PheAsO, phenylarsine oxide; DTE, 1.4-dithioerythritol; PLP, pyridoxal 5-phosphate; SITS, 4-acetamido-4′-isothiocyanato-2,2′-stilbenedisulfonic acid; DIDS, 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid; WRK, Woodward's reagent K; EDC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimmide; $C_{12}E_8$, octaethylene glycol monododecyl ether; NDP40, Nonidet P40

* Corresponding author. Dipartimento di Biologia Cellulare, Università della Calabria, Via P.Bucci cubo 4c, 87036 Arcavacata di Rende (CS), Italy. Tel.: +39 0984 492939; fax: +39 0984 492911.
E-mail address: indiveri@unical.it (C. Indiveri).

0005-2736/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2006.01.004

acyltransferase involved in the cholesterol metabolism [12] and internal carnitine acetyltransferase and carnitine palmitoyltransferase for the acetylation of intralumenal compounds, fatty acid elongation and other important functions [2,3,13,14]. Thus, as in the other organelles, the endoplasmic reticulum carnitine, may also play an important function in shuttling. Nevertheless, only very recently, the existence of a transport system for carnitine has been proposed on the basis of studies performed with intact microsomes [15]. The transport properties described for this transporter are different from those of the mitochondrial transporter [5,16], being more similar to the feature of a pore. The final proof of a protein mediated transport can be given by the reconstitution of the transporter into liposomes. This approach also allows the further characterization of the membrane transport system at a functional level and represents a methodology for testing the transport activity in purified protein fractions for molecular studies. This type of methodological approach has been used for carnitine transporters both of mitochondria and plasma membrane [16,17]. The present study investigated a procedure of the reconstitution of the carnitine transporter from solubilized rat liver microsomes into liposomes. Most of the functional properties found in the reconstituted system overlap with those described in intact microsomes, as well as additional functional properties of this transporter have been shown here.

2. Materials and methods

2.1. Materials

Amberlite Bio-Beads SM-2 was purchased from Bio-Rad, Sephadex G-50 and G-75 from Pharmacia, L-[^{3}H]carnitine and other labelled compounds from Amersham, L-α-phosphatidylcholine from fresh turkey egg yolk (EYPL) from Fluka. All other reagents were of analytical grade.

2.2. Preparation of microsomes

Microsomes were prepared from male Wistar rats. Livers were homogenized in an ice-cold medium containing 250 mM sucrose, 10 mM Tris, 1 mM EGTA (pH 7.4). The homogenate was centrifuged at 500×*g* for 10 min; the supernatant was then centrifuged at 10,000×*g* for 20 min; the resulting supernatant was centrifuged at 20,000×*g* for 20 min; the resulting supernatant was ultracentrifuged at 100,000×*g* for 60 min. The pellet was resuspended in fresh medium and centrifuged again at 100,000×*g* for 60 min. The microsomal pellet was then resuspended and stored at −80 °C.

2.3. Reconstitution of the carnitine transporter

Aliquots of 50 μl frozen microsomes (1–1.5 mg protein) were solubilized with 500 μl 1% Triton X-100 (without buffer and salts) and centrifuged for 10 min at 12,000×*g*. The protein extract was reconstituted into liposomes by the cyclic detergent removal procedure [18,19]. In this procedure, the mixed micelles containing detergent, protein and phospholipids were repeatedly passed through a column filled with Bio-Beads SM-2 resin to remove the detergent. The composition of the initial mixture used for reconstitution was: 50 μl of the microsomal extract (about 35 μg protein in 1% Triton X-100), 85 μl of 10% Triton X-114, 100 μl of liposomes (10 mg phospholipids, prepared as described previously [19]), 20 mM HEPES pH 8.0 (except when differently specified) and, when present, the internal substrate at the concentrations indicated in the figure legends, in a final volume of 700 μl. After vortexing, this mixture was passed 12 times through the same Bio-Beads SM-2 column (0.5×3.0 cm) preequilibrated with a buffer containing 20 mM HEPES pH 8.0. All the operations were performed at 4 °C, except the passages through Bio-Beads SM-2 column that were performed at room temperature.

2.4. Transport measurements

550 μl of proteoliposomes were passed through a Sephadex G-75 column (0.7×15 cm) preequilibrated with 20 mM NaCl and 10 mM HEPES (pH 8.0). The first 600 μl of the turbid eluate from the Sephadex column were collected, transferred to reaction vessels (100 μl each), and readily used for transport measurements by the inhibitor stop method [20]. For uptake measurements, transport was initiated by adding 0.1 mM [^{3}H]carnitine. After the required time interval, the reaction was stopped by adding 1 mM $HgCl_2$; in control samples, the inhibitor was added together with the labelled substrate at time zero. For efflux measurements, the proteoliposomes were prelabelled by transporter-mediated radioactivity equilibration before starting the transport assay. This was achieved by incubating the proteoliposomes (600 μl), passed through Sephadex G-75, with 10 mM [^{3}H]carnitine with a specific radioactivity of 10 nCi/nmol, for 60 min at 25 °C. Then, the external radioactivity was removed by passing again the proteoliposomes through Sephadex G-75 as described above. Transport was started by adding external buffer or substrates at the concentration indicated in the figure legends and stopped, at the appropriate time interval. The assay temperature was 25 °C. Finally, each sample of proteoliposomes (100 μl) was passed through a Sephadex G-50 column (0.6×8 cm) to separate the external from the internal radioactivity. The liposomes eluted with 1.3 ml of 20 mM NaCl were collected in 4 ml of scintillation mixture, vortexed and counted. For the determination of the [^{3}H]carnitine uptake, the experimental values were corrected by subtracting the respective controls (samples inhibited at time zero). For kinetic determinations, the initial transport rate was evaluated from the radioactivity taken up by the proteoliposomes in 5 min, i.e., within the initial linear range of the substrate uptake. Specific transport was referred to the amount of protein in the proteoliposomes. In the efflux experiments the transport activity was expressed as the residual intraliposomal cpm [19].

2.5. Other methods

The protein was determined by the Lowry method, modified for the presence of non-ionic detergents [21].

3. Results

3.1. Optimal conditions for reconstitution

The reconstitution procedure for the microsomal carnitine transporter has been optimized by adjusting the parameters that influence the efficiency of the solubilization of the transport protein in active form and its incorporation into the liposomes, i.e., the type and concentration of the detergent, the detergent/lipid ratio, the protein concentration and the number of passages through the same Bio-Beads SM-2 column [18,19]. In these experiments, the [^{3}H]carnitine uptake into the proteoliposomes in 10 min, that is related to the specific activity of the transporter, and the intraliposomal volume, that is related to the efficiency of reconstitution, have been measured.

For the reconstitution procedure used here, the protein has to be first solubilized in detergent and then mixed with phospholipids and additional detergent to allow the formation of proteoliposomes from mixed micelles (see Materials and methods). Various detergents that are efficiently absorbed onto Bio-Beads SM-2 were tested by using each of them both in the solubilization and the reconstitution steps. Triton X-114 was found to be the most effective; Triton X-100, $C_{12}E_8$, Triton N-57 and NDP40 led to a final transport activity ranging from

about 20 to 40% with respect to that obtained with Triton X-114 (Table 1). Taking into account the results described above, the effectiveness of adding a different detergent in the reconstitution step from that used for the solubilization was tested. It was found that the activity of the transporter was improved by solubilizing the microsomes with 1.0% Triton X-100 and adding Triton X-114 in the reconstitution mixture. The transport activity was optimal when the concentration of detergent in the reconstitution was 12 mg/ml Triton X-114; under these conditions the detergent/phospholipid ratio was 0.9 w/w (Table 1). No salt or buffer was added in the solubilization step, since these compounds reduced the extraction of the active transport protein (not shown).

The transport increased almost linearly with the protein concentration up to 40 μg/ml; above this value a saturation-like behaviour was observed. The intraliposomal volume decreased by increasing the protein concentration, indicating that higher concentrations of protein cause the formation of less or smaller liposomes (Fig. 1). The transport activity increased with the number of column passages up to 8 and remained nearly constant in the range from 8 to 18 passages; in this range the intraliposomal volume was not critically influenced by the number of columns passages (experiments not shown).

Table 1
Effect of detergents in the solubilization and reconstitution of the carnitine transporter

Detergent in solubilization (mg/ml)		Detergent in reconstitution (mg/ml)		Transport (pmol/mg/10 min)	Internal volume (μl/mg phospholipid)
TX-100	10	TX-100	8.6	34.2 ± 6.0	4.2 ± 0.4
		TX-100	12.0	56.0 ± 7.1	4.0 ± 0.4
		TX-114	5.7	59.6 ± 8.2	4.2 ± 0.3
		TX-114	8.6	144 ± 22	4.6 ± 0.4
		TX-114	12.0	242 ± 36	4.5 ± 0.5
		TX-114	15.7	212 ± 55	4.3 ± 0.4
		TX-114	20.0	193 ± 26	4.1±0.3
TX-114	10	TX-114	8.6	109±4.8	3.9±0.4
		TX-114	12.0	154±17	4.0±0.3
		TX-100	8.6	47.5±7.6	4.1±0.4
		TX-100	12.0	86.4±12	3.7±0.4
$C_{12}E_8$	10	$C_{12}E_8$	12.0	56.6±9.4	2.8±0.3
		TX-100	12.0	39.6±5.4	3.1±0.3
		TX-114	12.0	58.0±7.2	3.7±0.3
TN-57	10	TN 57	12.0	44.4±6.2	2.1±0.2
		TX-100	12.0	47.0±7.4	2.7±0.3
		TX-114	12.0	58.0±8.4	3.5±0.3
NDP40	10	NDP40	12.0	23.8±6.0	2.5±0.2
		TX-100	12.0	30.2±5.8	2.6±0.3
		TX-114	12.0	37.2±10	3.1±0.2

Microsomes were solubilized, with a buffer containing 1% of the indicated detergent. The reconstitution was performed, as described in Materials and methods, except that the different detergents at the indicated concentrations were added. Transport and internal volume of the proteoliposomes was measured as described in Materials and methods. The results are means±S.D. from three experiments.
Abbreviation: T = Triton.

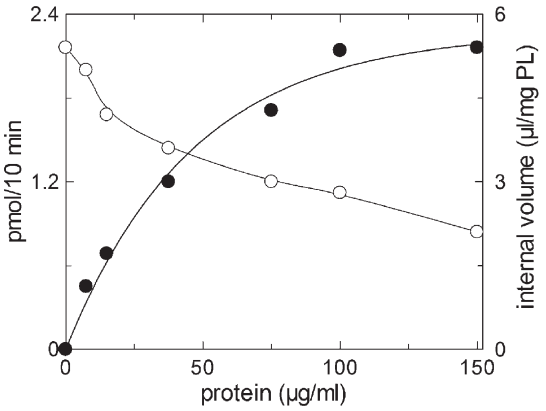

Fig. 1. Dependence of the carnitine transport activity and proteoliposomal volume on protein concentration. The reconstitution was performed as described in Materials and methods except that the concentrations of protein in the reconstitution mixture was varied as indicated. Transport was measured as 0.1 mM [^{3}H]carnitine uptake into proteoliposomes, (●); internal volume of the proteoliposomes, (○). Similar results were obtained in three different experiments.

According to the results described, a protein concentration of about 50 μg/ml (corresponding to a protein/phospholipid ratio of 0.005 w/w), which represents a good compromise between optimal activity and internal volume, and 12 column passages were used in the experiments.

Cardiolipin has been reported to be essential for the activity of the mitochondrial carnitine carrier [22]. This phospholipid did not exert any influence on the activity of the reconstituted microsomal carnitine transporter upon addition to the solubilization or the reconstitution at concentrations ranging from 20 to 200 μg/ml (not shown).

3.2. Functional characterization

To investigate the mode of transport (i.e., uniport or antiport) of the reconstituted transporter, the time dependence of carnitine uptake into proteoliposomes has been studied in the presence and absence of internal substrate. To achieve this objective, 0.1 mM [^{3}H]carnitine was added to proteoliposomes without internal substrate or containing internal 15 mM carnitine or acetylcarnitine. As shown in Fig. 2, the uptake of [^{3}H]carnitine into the proteoliposomes with or without internal carnitine or acetylcarnitine was nearly the same, increasing with the time up to the radioisotopic equilibrium that was reached after about 60 min. The data indicated that the transport process occurred by a uniport mode. The transport was independent of the presence of external or internal Na^+ (not shown) excluding the occurrence of co-transport. The experimental data describing the transport of carnitine in the absence of intraliposomal substrate, fitted a first order rate equation. The first order rate constant, k, was 0.068 min^{-1} (0.080±0.023 from 3 different experiments); the transport rate (calculated as the product of k and the transport at equilibrium) was 28 pmol × min^{-1} × mg $protein^{-1}$ (26±5 from 3 different experiments). The carnitine uptake was abolished by the incubation of the proteoliposomes with trypsin. Furthermore no carnitine uptake was detected into proteoliposomes

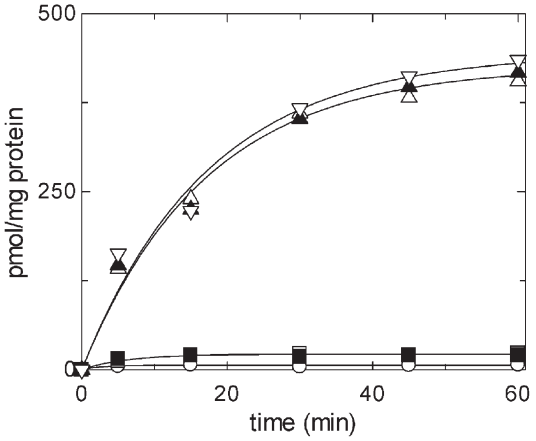

Fig. 2. Time course of [^{3}H]carnitine uptake by reconstituted proteoliposomes. 0.1 mM [^{3}H] carnitine was added at time zero to proteoliposomes containing 15 mM internal carnitine (▲), or 15 mM internal acetylcarnitine (▽) or without internal substrate (△). (□) 0.2 mg/ml trypsin was added to proteoliposomes without internal substrate; (○) liposomes reconstituted without protein or (■) with boiled protein; the transport reaction was stopped at the indicated times, as described in Materials and methods. Similar results were obtained in three different experiments.

reconstituted with boiled protein or without protein. These data indicated that the [^{3}H]carnitine uptake observed was due to the solubilized protein.

The effect, on the carnitine transport, of protein modifying reagents that react with lysine (PLP, DIDS, SITS [23,24]), cysteine (maleimides, mersalyl, $HgCl_2$ PheAsO [25,26]), arginine (methylglyoxal, phenylglyoxal [27]), and glutamate or aspartate (WRK [28], EDC [24]) functional groups was tested. As shown in Table 2, among the SH reagents, only $HgCl_2$ efficiently inhibited the transport leading to a suppression of about 80% of the activity at 0.5 mM concentration. Among the reagents of other functional groups, PLP inhibited 50% of the transport although only at considerably higher concentrations and DIDS exerted a significant inhibition (70%) at 1 mM concentration. All the other inhibitors had very low if any effect. As $HgCl_2$ was the most effective inhibitor, the inhibition was further characterised. The dependence of the inhibition on the concentration of $HgCl_2$ was studied. The data is reported in Fig. 3 A as a dose–response curve, from which an IC50 of 0.17 mM (0.16±0.03 mM) for $HgCl_2$ was calculated. In order to verify whether $HgCl_2$ reacted with Cys residues of the protein, the effect of DTE on the inhibition was tested. As expected (Fig. 3B), $HgCl_2$ strongly reduced the uptake of carnitine in the proteoliposomes. On the contrary, DTE had no effect on the uptake. After 20 min the addition of DTE to the proteoliposomes treated with $HgCl_2$ rapidly and completely reversed the inhibition, indicating that $HgCl_2$ inhibited the transporter by reacting with SH group(s) of Cys residues of the protein.

The specificity of the transporter towards molecules that have some structural homology with carnitine or that are known to be transported through intracellular membranes like

Table 2
Effect of inhibitors on the reconstituted carnitine transport

Inhibitor	Concentration (mM)	Residual activity (%)
NEM	1	98±8
NPheM	1	103±12
mersalyl	1	95±11
$HgCl_2$	0.5	21±8
PheAsO	1	96±9
methylglyoxal	5	98±5
phenylglyoxal	5	100±12
WRK	5	108±11
EDC	2	95±9
PLP	20	50±10
SITS	2	102±9
DIDS	1	30±8

Transport was measured as 0.1 mM [^{3}H] carnitine uptake in 10 min into proteoliposomes, reconstituted as described in Materials and methods. The inhibitors were added 1 min before the labelled substrate at the concentrations indicated. Percent of residual activity was calculated for each experiment with respect to the control sample (without added inhibitor, referred as 100%). The results are means±S.D. of the percentages for three experiments. The average transport activity of the control samples of the three experiments analyzed was 279±67 pmol/10 min/mg protein.

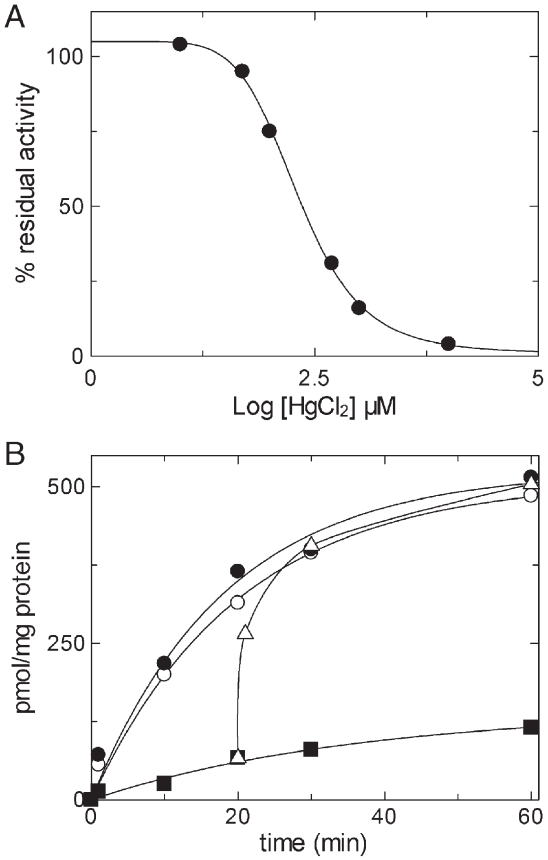

Fig. 3. Inhibition of the carnitine transporter by $HgCl_2$ and reversal by DTE. (A), dose response curve for the inhibition of the reconstituted carnitine transport by $HgCl_2$. Proteoliposomes were incubated with $HgCl_2$ at the indicated concentrations for 1 min. Then, carnitine transport was measured by adding 0.1 mM [^{3}H] carnitine and stopped after 5 min, as described in Materials and methods. (B) Time course of [^{3}H]carnitine uptake by reconstituted proteoliposomes (○) in the absence or (●) in the presence of DTE, and in the presence of 0.5 mM $HgCl_2$ (■). In (△) 3 mM DTE was added to an aliquot of proteoliposomes treated with $HgCl_2$.

Table 3
Specificity of the reconstituted transporter towards substrates

Labelled substrate	Transport (pmol/10 min/mg protein)
[^{3}H] carnitine	270±42
[^{3}H] ornithine	202±75
[^{3}H] lysine	150±42
[^{3}H] arginine	177±21
[^{3}H] glutamine	195±21
[^{14}C] glutamate	77±52
[^{14}C] malate	42±27
[^{14}C] citrate	47±31
[^{32}P] ATP	30±22

Transport was measured as uptake of 0.1 mM of the indicated labelled substrates in 10 min into proteoliposomes, reconstituted as described in Materials and methods. The results are means±S.D. from three experiments.

endoplasmic reticulum or mitochondrial membranes [29,30] were tested. To investigate this further, the transport of different labelled substrates was determined in proteoliposomes reconstituted with the microsomal extract under the condition of optimal carnitine transport activity. As shown in Table 3, besides carnitine, which was the most efficiently transported substrate, also ornithine, glutamine, arginine, lysine were transported by the reconstituted protein, however with a lower efficiency. On the contrary, glutamate, malate, citrate and ATP showed a very low transport activity.

The dependence of the transport rate on the carnitine concentration was studied (Fig. 4). The data fitted the Michaelis–Menten equation from which a Km of 11.7 mM (10.9±0.16 mM from 5 exp.) and a V_{max} of 0.77 nmol/min/mg protein (0.70±0.26 nmol/min/mg from 5 exp.) was extrapolated. The relatively high standard deviation often associated with the rate values is due to variations in the amount of active transport protein molecules present in the different preparations.

The dependence of the transport rate on the temperature was studied. The Arrhenius plot of the experimental data (Fig. 5) showed a linear dependence in the range 10–32 °C. From the slope of the straight line, an activation energy of 16.1 kJ/mol was calculated. Unexpectedly, the transport rate increased at temperatures below 10 °C (not shown).

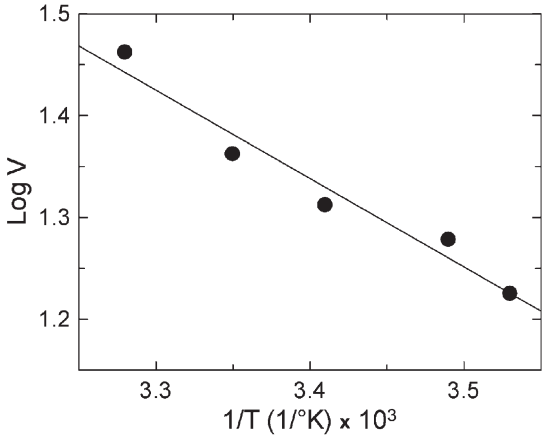

Fig. 5. Arrhenius plot of the temperature dependence of the reconstituted carnitine transport activity. 0.1 mM [^{3}H] carnitine was added to proteoliposomes incubated at the indicated temperature. The transport activity (v) is expressed as pmol/min/mg protein. Similar results were obtained in two different experiments.

The dependence of the transport activity on the pH was also studied. Fig. 6 shows the dependence of the initial rate and the transport at the equilibrium in a range of pH between pH 6.5 and pH 9.0. The maximal activity was measured at pH 8.0. A sharp decrease of transport was observed at a lower pH, whereas a less dramatic decrease was detected at a more alkaline pH in respect to pH 8.0. Similar patterns were observed both at transport rate and equilibrium transport indicating that the pH may influence both the activity of the transport protein and the amount of transporter inserted into the liposomes.

To verify whether the reconstituted transporter could also catalyze a carnitine flux from inside to outside the proteoliposomes, the carnitine efflux was determined. To this aim, the

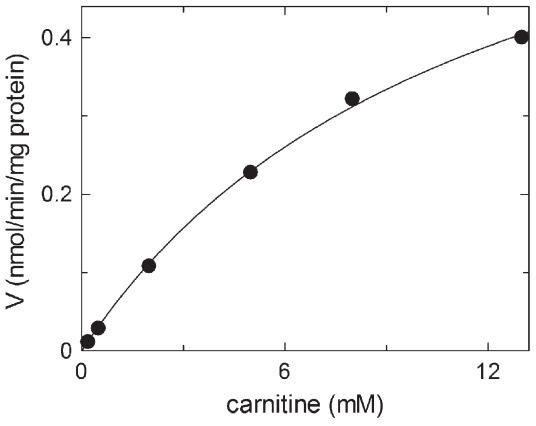

Fig. 4. Dependence on substrate concentration of the rate of carnitine transport. [^{3}H] carnitine at the indicated concentration was added to proteoliposomes. The transport rate was measured, as described in Materials and methods as uptake in 5 min. Data were plotted according to Michaelis–Menten. Similar results were obtained in five different experiments.

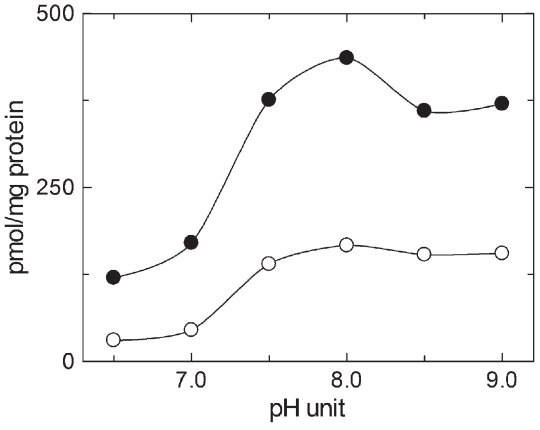

Fig. 6. Effect of pH on the reconstituted carnitine transporter. All the experimental procedures from the reconstitution to the transport measurement (see Materials and methods) were performed in 20 mM HEPES/Tris buffer at the indicated pH. Transport rate was measured as 0.1 mM [^{3}H] carnitine uptake in 5 min (○) or 60 min (●) into proteoliposomes. Similar results were obtained in three different experiments.

proteoliposomes were prelabelled by the transporter-mediated process (see Materials and methods) that allows the accumulation of radioactivity only into the active proteoliposomes, i.e., vesicles containing active transporter molecules in the membrane. Furthermore, by this procedure, it was also possible to check whether longer chain acylcarnitine could stimulate the carnitine counter-transport. In fact, this type of experiment was not allowed with long chain acylcarnitine inside the vesicles, since these molecules exert a detergent effect [31]. Fig. 7 shows that the labelled carnitine effluxed from the reconstituted vesicles. The efflux was virtually abolished by the addition of $HgCl_2$ to the proteoliposomes; nearly no inhibition was observed upon addition of mersalyl (not shown). The addition of 10 mM carnitine, 10 mM acetylcarnitine, 10 mM propionylcarnitine (not shown), 10 mM ornithine (not shown), 2.5 mM pentanoylcarnitine or 2 mM octanolylcarnitine, to the external side of the vesicles, did not exert any effect on the basic efflux. On the contrary, the addition of 0.5 mM stearoylcarnitine or 0.5 mM palmitoylcarnitine, that could not be used at higher concentrations due to their detergent property, stimulated the carnitine efflux. The finding that both the uptake (prelabelling; see Materials and methods) and the efflux processes occurred in the same active proteoliposome pool and were inhibited by $HgCl_2$, indicated that they were mediated by the same transporter.

4. Discussion

A definitive proof of the existence of a membrane transporter can be given by its functional reconstitution into artificial membrane. The reconstitution system leads to further advantages in the functional characterization of a transporter, with respect to intact cells or sub-cellular membrane vesicles, such as the possibility to control the experimental conditions in the internal compartment; the reduction of interferences due to the virtual absence of external and internal enzymes; a longer radioactivity equilibration time with respect to native membrane vesicles, due to the low protein/lipid ratio (less that one protein per liposome), which leads to a better kinetic resolution and the possibility of modifying the lipid composition of the membrane. In this paper, the existence of the carnitine transporter of the endoplasmic reticulum, previously proposed on the basis of studies in intact microsomes [15], has been confirmed by its reconstitution into liposomes. We have adopted a reconstitution procedure successfully used for the characterization of mitochondrial and plasma membrane transport systems [17,19,32] among which are the mitochondrial and the plasma membrane (OCTN2) carnitine transporters [16,17]. This reconstitution method leads to the formation of proteoliposomes larger than those obtained by other methods like the freeze–thaw sonication [19] thus increasing the accumulation of radioactivity inside the active vesicles, i.e., the proteoliposomes containing active transporters inserted into the membrane. The reconstitution has been optimized by adjusting the critical factors influencing the formation of the proteoliposomes, i.e., the type and concentration of detergent, the detergent/phospholipid ratio, the protein/phospholipid ratio and the number of passages through the Bio-Beads SM-2 column. Differently from the reconstitution of other carnitine transporters carried out by this procedure [16,17], the optimal condition for the reconstitution of the endoplasmic reticulum carnitine transporter has been achieved by using different detergents in the solubilization and reconstitution steps. This difference in the reconstitution parameters reflects the different functional properties of this transporter with respect to the mitochondrial and plasma membrane carnitine transport systems (see below). To our knowledge, the reconstitution of the carnitine transporter represents one of the few cases of reconstitution of transport systems of the endoplasmic reticulum. The sulfate transporter was reconstituted by freeze–thaw sonication [33]; the ATP transporter was reconstituted by a procedure based on detergent dilution [29]. Very recently a transporter for mannosylphosphoryldolichol analogues and the GDP-mannose transporter have been reconstituted by a dialysis procedure [34] and by detergent removal/freeze–thaw sonication [35] respectively.

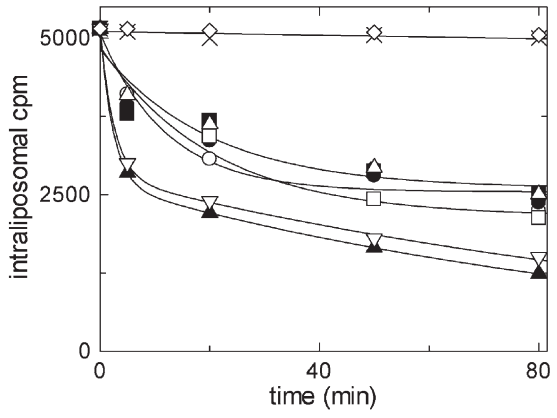

Fig. 7. Dependence of [^{3}H] carnitine efflux from proteoliposomes on external carnitine derivatives. Efflux of 15 mM [^{3}H] carnitine was measured in the presence of external 10 mM HEPES pH 8, 20 mM NaCl: (○) no addition, (●) 10 mM carnitine, (△) 10 mM acetylcarnitine, (■) 2.5 mM pentanoylcarnitine, (□) 2 mM octanoylcarnitine, (▽) 0.5 mM palmitoylcarnitine (▲) 0.5 mM stearoylcarnitine, (x) 0.5 mM $HgCl_2$ and (◇) 0.5 mM palmitoylcarnitine plus 0.5 mM $HgCl_2$. Similar results were obtained in three different experiments in which the initial intraliposomal radioactivity ranged from about 3800 to 5600 cpm.

The reconstitution of the carnitine transport activity described here indicates the occurrence of a protein mediated process, that is confirmed by the following findings: the treatment of the proteoliposomes with trypsin and boiling abolish the transport of carnitine; reagents that specifically react with Cys ($HgCl_2$) and Lys (PLP and DIDS) residues, inhibit the carnitine uptake. On the one hand, the inhibition exerted by DIDS seems to be a common property of endoplasmic reticulum transport systems [29,33–37]. On the other hand, it has been found that the inhibition caused by $HgCl_2$ is a specific property of the carnitine transporter; thus, it has been investigated further. The inhibition by $HgCl_2$ is due to its reaction with one or more Cys residues of the protein as was confirmed by the fast reversal when DTE was added. The finding that other SH reagents like mersalyl or NEM fail to inhibit the transport, suggests that the critical Cys residues must be located in a small hydrophilic pocket of the protein that can be reached by $HgCl_2$ (ranging in

size from 1 Å of the Hg^{++} to 2.4 Å of the $HgCl_2$ [38]) and not by other larger compounds. It can also be hypothesized that two close SH residues must be cross-linked to inhibit the transport; accordingly, $HgCl_2$, differently from NEM or mersalyl, can simultaneously bind two vicinal SH residues [26].

The mode of transport of the reconstituted carnitine transporter is of the uniport type. However it was found that the externally added long chain carnitine derivatives specifically stimulate the efflux of carnitine from the proteoliposomes. The most probable interpretation of this result is that the same transporter that catalyses the uniport of carnitine may behave as an antiporter carnitine/long chain acylcarnitine. This is in agreement with the function of the carnitine shuttle in the endoplasmic reticulum in which the transfer of cytosolic long chain acyl-CoA is essential for the diacylglycerol synthesis used to assemble the VLDL. The antiport may also function the other way round, i.e., carnitine from inside to outside the proteoliposomes and acylcarnitine in the opposite direction. However, this condition could not be experimentally tested, since the long chain acylcarnitines exerted a strong detergent effect when they were inserted inside the vesicles during the reconstitution procedure. Additionally, the carnitine uniport should play the function of equilibrating the endoplasmic reticulum and the cytosolic carnitine concentration. The property to catalyze both antiport and uniport of substrates has been previously described for the mitochondrial carnitine/acylcarnitine transporter [39], even though with some differences with respect to the endoplasmic reticulum transporter. Furthermore, the ability to catalyze both uniport and antiport of substrates may explain the previous results obtained in intact microsome studies [15]. The carnitine transporter of the endoplasmic reticulum shows specificity towards substrates that share the presence of positive and negative charges in the molecule; whereas negatively charged substrates are transported with a very low efficiency or are not transported. Thus, the carnitine transporter may also function as an amino acid transporter, as was found for the yeast plasma membrane carnitine transporter Agp2p [9,40]. In the reconstituted system it was determined the Km of the transporter for carnitine; its value is higher than the carnitine concentration in the cell and it is of the same range of Km as the transporter for mannosylphosphoryldolichol [34]. There is no experimental evidence to assess if the transporter has inserted into the proteoliposomes in the native orientation. Since several transport systems reconstituted by the cyclic detergent removal procedure, that has been used in this study, showed a right side out orientation [17–19,30,32], it is very likely that the endoplasmic reticulum carnitine transporter also inserted in a similar way. Thus, the Km measured in the proteoliposomes should correspond to the extralumenal Km. The activation energy measured for the reconstituted transporter is lower than that of the carriers [16]. Thus, the endoplasmic carnitine transporter seems to share carrier and channel properties as well. From a functional point of view, the carnitine transporter of the endoplasmic reticulum is quite different from the carnitine transporters of mitochondria and plasma membrane (OCTN2). On the one hand, the transporter described in this paper, shows different sensitivity to inhibitors, a low affinity for carnitine, a lack of dependence on cardiolipin and a low activation energy that unequivocally distinguishes it from the mitochondrial ones [16]. On the other hand the OCTN2 transporter is clearly distinguished from the endoplasmic reticulum transporter by the obligatory antiport mode of transport, the sodium dependence, the very different affinity for carnitine and the pH dependence [17,41]. On the basis of these observations it may be hypothesized that the endoplasmic reticulum carnitine transporter does not belong either to the mitochondrial carrier family or to the OCTN sub-family of the plasma membrane. At this stage it cannot be said whether the endoplasmic reticulum carnitine transporter belongs to a known protein family or it is a single protein member as was found, for example, in the case of the CorA magnesium transport system [42]. This last possibility has to be taken into account in view of the peculiar functional properties of the transport system described here. A screening of the protein databases revealed the existence of sequences with unknown functions that show some percentage of endoplasmic reticulum localization, as analyzed by the PSORT software. However, the identity of these sequences with proteins involved in carnitine transport and metabolism, found by global and local alignment analysis, did not give significant indications to identify the hypothetical sequence of the carnitine transporter. However, it seems very likely that there are still enough unidentified protein sequences and most likely untranslated genes that may be the focus for further studies into the identification of the molecular counterpart of the endoplasmic reticulum carnitine transporter.

Acknowledgements

This work has been supported by a grant from MIUR-FIRB (Ministero dell'Istruzione, dell'Università e della Ricerca-Fondo per gli Investimenti della Ricerca di Base) n.RBAU01-H33S.The authors are indebted to Dr. Arduino Arduini for the initial valuable discussions on the microsomal carnitine shuttle.

References

[1] L.L. Bieber, Carnitine, Annu. Rev. Biochem. 57 (1988) 261–283.
[2] R.R. Ramsay, A. Arduini, The carnitine acyltransferases and their role in modulating acyl-CoA pools, Arch. Biochem. Biophys. 302 (1993) 307–314.
[3] R.R. Ramsay, R.D. Gandour, F.R. van der Leij, Molecular enzymology of carnitine transfer and transport, Biochim. Biophys. Acta 1546 (2001) 21–43.
[4] J. Kerner, C. Hoppel, Fatty acid import into mitochondria, Biochim. Biophys. Acta 1486 (2000) 1–17.
[5] S.V. Pande, M.S. Murthy, Carnitine-acylcarnitine translocase deficiency: implications in human pathology, Biochim. Biophys. Acta 1226 (1994) 269–276.
[6] V.A. Zammit, Carnitine acyltransferases: functional significance of subcellular distribution and membrane topology, Prog. Lipid Res. 38 (1999) 199–224.
[7] V.A. Zammit, The malonyl-CoA-long-chain acyl-CoA axis in the maintenance of mammalian cell function, Biochem. J. 343 (1999) 505–515.
[8] V.A. Zammit, F. Fraser, Submitochondrial and subcellular distributions of the carnitine-acylcarnitine carrier, FEBS Lett. 445 (1999) 41–44.
[9] C.W. van Roermund, E.H. Hettema, M. van den Berg, H.F. Tabak, R.J. Wanders, Molecular characterization of carnitine-dependent transport of

acetyl-CoA from peroxisomes to mitochondria in *Saccharomyces cerevisiae* and identification of a plasma membrane carnitine transporter, Agp2p, EMBO J. 8 (1999) 5843–5852.
[10] M.R. Owen, C.C. Corstorphine, V.A. Zammit, Overt and latent activities of diacylglycerol acyltransferase in rat liver microsomes: possible roles in very-low-density lipoprotein triacylglycerol secretion, Biochem. J. 323 (1997) 17–21.
[11] K.A. Abo-Hashema, M.H. Cake, G.W. Power, D. Clarke, Evidence for triacylglycerol synthesis in the lumen of microsomes via a lipolysis-esterification pathway involving carnitine acyltransferases. 274 (1999) 35577–35582.
[12] C.W. Joyce, G.S. Shelness, M.A. Davis, R.G. Lee, K. Skinner, R.A. Anderson, L.L. Rudel, ACAT1 and ACAT2 membrane topology segregates a serine residue essential for activity to opposite sides of the endoplasmic reticulum membrane, Mol. Biol. Cell 11 (2000) 3675–3687.
[13] F. Fraser, C.G. Corstorphine, N.T. Price, V.A. Zammit, Evidence that carnitine palmitoyltransferase I (CPT I) is expressed in microsomes and peroxisomes of rat liver. Distinct immunoreactivity of the N-terminal domain of the microsomal protein, FEBS Lett. 446 (1999) 69–74.
[14] J. Tiralongo, H. Schmid, R. Thun, M. Iwersen, R. Schauer, Characterisation of the enzymatic 4-O-acetylation of sialic acids in microsomes from equine submandibular glands, Glycoconj. J. 17 (2000) 849–858.
[15] J.M. Gooding, M. Shayeghi, E.D. Saggerson, Membrane transport of fatty acylcarnitine and free L-carnitine by rat liver microsomes, Eur. J. Biochem. 271 (2004) 954–961.
[16] C. Indiveri, A. Tonazzi, G. Prezioso, F. Palmieri, Kinetic characterization of the reconstituted carnitine carrier from rat liver mitochondria, Biochim. Biophys. Acta 1065 (1991) 231–238.
[17] L. Pochini, F. Oppedisano, C. Indiveri, Reconstitution into liposomes and functional characterization of the carnitine transporter from renal cell plasma membrane, Biochim. Biophys. Acta 1661 (2004) 78–86.
[18] R. Krämer, C. Heberger, Functional reconstitution of carrier proteins by removal of detergent with a hydrophobic ion exchange column, Biochim. Biophys. Acta 863 (1986) 289–296.
[19] F. Palmieri, C. Indiveri, F. Bisaccia, V. Iacobazzi, Mitochondrial metabolite carrier proteins: purification, reconstitution, and transport studies, Methods Enzymol. 260 (1995) 349–369.
[20] F. Palmieri, M. Klingenberg, Direct methods for measuring metabolite transport and distribution in mitochondria, Methods Enzymol. 56 (1979) 279–301.
[21] J.R. Dulley, P.A. Grieve, A simple technique for eliminating interference by detergents in the Lowry method of protein determination, Anal. Biochem. 64 (1975) 136–141.
[22] C. Indiveri, A. Tonazzi, F. Palmieri, Identification and purification of the carnitine carrier from rat liver mitochondria, Biochim. Biophys. Acta 1020 (1990) 81–86.
[23] R. Stappen, T. Dierks, A. Broer, R. Kramer, Probing the active site of the reconstituted aspartate/glutamate carrier from mitochondria. Structure/function relationship involving one lysine and two cysteine residues, Eur. J. Biochem. 210 (1992) 269–277.
[24] T. Dierks, R. Stappen, A. Salentin, R. Kramer, Probing the active site of the reconstituted aspartate/glutamate carrier from bovine heart mitochondria: carbodiimide-catalyzed acylation of a functional lysine residue, Biochim. Biophys. Acta 1103 (1992) 13–24.
[25] A. Tonazzi, C. Indiveri, Chemical modification of the mitochondrial ornithine/citrulline carrier by SH reagents: effects on the transport activity and transition from carrier to pore-like function, Biochim. Biophys. Acta 1611 (2003) 123–130.
[26] P.R. van Iwaarden, A.J. Driessen, W.N. Konings, What we can learn from the effects of thiol reagents on transport proteins, Biochim. Biophys. Acta 1113 (1992) 161–170.
[27] T. Oya, N. Hattori, Y. Mizuno, S. Miyata, S. Maeda, T. Osawa, K. Uchida, Methylglyoxal modification of protein. Chemical and immunochemical characterization of methylglyoxal-arginine adducts, J. Biol. Chem. 274 (1999) 18492–18502.
[28] A.J. Brake, B.H. Weber, Evidence for an essential carboxyl group for yeast phosphoglycerate kinase. Reaction with Woodward's reagent K, J. Biol. Chem. 249 (1974) 5452–5457.
[29] E. Guillen, C.B. Hirschberg, Transport of adenosine triphosphate into endoplasmic reticulum proteoliposomes, Biochemistry 34 (1995) 5472–5476.
[30] F. Palmieri, The mitochondrial transporter family (SLC25): physiological and pathological implications, in: M.A. Hediger (Ed.), The ABC of Solute Carriers, Pflugers Arch.-Eur. J. Physiol., vol. 447, 2004, pp. 689–709.
[31] C. Indiveri, V. Iacobazzi, N. Giangregorio, F. Palmieri, Bacterial overexpression, purification, and reconstitution of the carnitine/acylcarnitine carrier from rat liver mitochondria, Biochem. Biophys. Res. Commun. 249 (1998) 589–594.
[32] F. Oppedisano, L. Pochini, M. Galluccio, M. Cavarelli, C. Indiveri, Reconstitution into liposomes of the glutamine/amino acid transporter from renal cell plasma membrane: functional characterization, kinetics and activation by nucleotides, Biochim. Biophys. Acta 1667 (2004) 122–131.
[33] I. Sekler, RS. Lo, T. Mastrocola, RR. Kopito, Sulfate transport mediated by the mammalian anion exchangers in reconstituted proteoliposomes, J. Biol. Chem. 270 (1995) 11251–11256.
[34] J.S. Rush, C.J. Waechter, Functional reconstitution into proteoliposomes and partial purification of a rat liver ER transport system for a water-soluble analogue of mannosylphosphoryldolichol, Biochemistry 43 (2004) 7643–7652.
[35] H. Segawa, R.P. Soares, M. Kawakita, S.M. Beverley, S.J. Turco, Reconstitution of GDP-mannose transport activity with purified Leishmania LPG2 protein in liposomes, J. Biol. Chem. 280 (2005) 2028–2035.
[36] G. Banhegyi, L. Lusini, F. Puskas, R. Rossi, R. Fulceri, L. Braun, V. Mile, P. di Simplicio, J. Mandl, A. Benedetti, Preferential transport of glutathione versus glutathione disulfide in rat liver microsomal vesicles, J. Biol. Chem. 274 (1999) 12213–12216.
[37] M.A. Zoccoli, R.R. Hoopes, M.L. Karnovsky, Identification of a rat liver microsomal polypeptide involved in the transport of glucose 6-phosphate. Labeling with 4,4′-diisothiocyano-1,2-diphenyl[3H]ethane-2,2′-disulfonic acid, J. Biol. Chem. 257 (1982) 3919–3924.
[38] A. Tonazzi, N. Giangregorio, F. Palmieri, C. Indiveri, Relationships of Cysteine and Lysine residues with the substrate binding site of the mitochondrial ornithine/citrulline carrier: an inhibition kinetic approach combined with the analysis of the homology structural model, Biochim. Biophys. Acta 1718 (2005) 53–60.
[39] C. Indiveri, A. Tonazzi, F. Palmieri, Characterization of the unidirectional transport of carnitine catalyzed by the reconstituted carnitine carrier from rat liver mitochondria, Biochim. Biophys. Acta 1069 (1991) 110–116.
[40] J.L. Schreve, J.M. Garrett, Yeast Agp2p and Agp3p function as amino acid permeases in poor nutrient conditions, Biochem. Biophys. Res. Commun. 313 (2004) 745–751.
[41] I. Tamai, R. Ohashi, J. Nezu, Y. Yabuuchi, A. Oku, M. Shimane, Y. Sai, A. Tsuji, Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2, J. Biol. Chem. 273 (1998) 20378–20382.
[42] R.L. Smith, J.L. Banks, M.D. Snavely, M.E. Maguire, Sequence and topology of the CorA magnesium transport systems of *Salmonella typhimurium* and *Escherichia coli*. Identification of a new class of transport protein, J. Biol. Chem. 268 (1993) 14071–14080.