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Identification and purification of the carnitine carrier from rat liver mitochondria

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The carnitine carrier from rat liver mitochondria, solubilized in Triton X-100 and partially purified on hydroxyapatite, was identified and completely purified by specific elution from celite in the presence of cardiolipin. On SDS-gel electrophoresis, the purified celite fraction consisted of a single band with an apparent M_r of 32500. When reconstituted into liposomes the carnitine transport protein catalyzed an *N*-ethylmaleimide-sensitive carnitine / carnitine exchange. It was purified 970-fold with a recovery of 43% and a protein yield of 0.04% with respect to the mitochondrial extract. The properties of the reconstituted carrier, i.e., requirement for a countersubstrate, substrate specificity and inhibitor sensitivity, were similar to those of the carnitine transport system as characterized in intact mitochondria.

Introduction

Most of the enzymes connected with carnitine metabolism and function have been characterized and purified in the recent years [1]. Nevertheless, little is known so far about the transport systems for carnitine or carnitine derivatives present in biological membranes. Among these, the system most investigated is the mitochondrial carnitine carrier, which has a central role in the transfer of the fatty acids as acylcarnitines into the mitochondrial matrix, where the acyl groups are released to be used for fatty acid oxidation.

The main properties of this carrier have been studied in intact mitochondria [2–8]. It catalyzes the transport of carnitine and acylcarnitines of various lengths by an exchange mechanism [8]; it is inhibited by SH-reagents and some analogues of acylcarnitines like sulphobetaines [4,5].

However, essential for the identification of a transport protein and for its detailed functional and structural characterization is the purification and reconstitution of the purified protein in artificial membranes.

The carnitine carrier has previously been reconstituted from a total mitochondrial extract by Noel et al. [9] and has been partially purified by us [10].

We describe here the purification and the identification of the mitochondrial carnitine carrier. The functional properties of the reconstituted transport activity resemble those known from intact mitochondria [1,11]. Additional aspects of this carrier with respect to inhibitor sensitivity are shown in this paper.

Materials and Methods

Materials. Hydroxyapatite (Bio Gel HTP) and Affi-Gel 501 were purchased from Bio-Rad, Silica-gel 60 and Fractogel TSK AF Brown from Merk, celite 535 from Roth, Amberlite XAD-2 from Fluka, Sephadex G-50 and G-75 from Pharmacia, L-[^{3}H]carnitine and 2-[1-^{14}C]oxoglutarate from Amersham, phospholipids (phosphatidylcholine from fresh turkey egg yolk), cardiolipin, Pipes, Triton X-100, L-carnitine from Sigma, L-octanoylcarnitine, L-palmitoylcarnitine from Serva, *n*-octyl-, *N*-decyl-, *N*-dodecyl-, *N*-tetradecyl- and *N*-hexadecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulphonate from Calbiochem. All other reagents were of analytical grade.

Purification of the carnitine carrier. Rat liver mitochondria, prepared as described in Ref. 12, were solubilized in 3% Triton X-100 (w/v)/50 mM NaCl/1 mM DTE/10 mM Pipes (pH 7) (16–18 mg protein/ml), and centrifuged at 100000 × *g* for 15 min at 4°C. 0.6 ml of the supernatant (extract) were applied to a 1.5 g dry hydroxyapatite column (0.7 cm diameter) and eluted with 3% Triton X-100/20 mM Na_2SO_4/1 mM DTE/10 mM Pipes (pH 7) at 0.05 ml per min. The first 0.8 ml

Abbreviations: DTE, dithioerythritol; HTP, hydroxyapatite; Pipes, 1,4-piperazinediethanesulphonic acid; SDS, sodium dodecylsulphate.

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were collected (HTP eluate). 0.5 ml of the HTP eluate were applied onto a dry celite column (0.25 g, 0.5 cm diameter) and eluted with: 0.5 ml of 3% Triton X-100/10 mM Na_2SO_4/10 mM Pipes (pH 7), 0.5 ml of 3% Triton X-100/5 mM Pipes (pH 7), and 1.0 ml of 3% Triton X-100/10 mM Pipes (pH 7) plus 1.6 mg/ml cardiolipin; all these buffers contained 1 mM DTE. Fractions of 0.5 ml were collected.

Reconstitution of the carnitine carrier in the liposomes. Protein eluates were reconstituted by removing the detergent with a hydrophobic ion-exchange column [13,14]. In this procedure, the mixed micelles containing detergent, protein and phospholipids, were repeatedly passed through Amberlite XAD columns. The composition of the mixture used for reconstitution was: 200 μl of the HTP or celite eluates or 30 μl of the extract, 15 μl of cardiolipin (40 mg/ml), 60 μl of 10% Triton X-100, 100 μl of 10% egg yolk phospholipids in the form of sonicated liposomes prepared as described in Ref. 15, 20 mM L-carnitine or alternatively 20 mM 2-oxoglutarate and 50 mM sodium phosphate (pH 7) in a final volume of 680 μl. After vortexing, this mixture was passed 15 times through the same Amberlite column (0.5 × 3.6 cm), preequilibrated with a buffer containing 50 mM sodium phosphate (pH 7) and 20 mM carnitine or 20 mM 2-oxoglutarate. All the operations were performed at 4 °C, except for the passage through Amberlite, carried out at room temperature.

Transport measurements. In order to remove the external substrate 550 μl of proteoliposomes were passed through a Sephadex G-75 column (0.7 × 15 cm) preequilibrated with 50 mM NaCl/10 mM Pipes (pH 7). The eluted proteoliposomes (700 μl), distributed in reaction vessels (100 μl), were used for transport measurements by the inhibitor stop method [16]. Transport was started by adding 10 μl of 1.1 mM [^{3}H]carnitine or 2-[^{14}C]oxoglutarate (about 200000 dpm/sample) and stopped after 10 min by adding 5 μl of 30 mM *N*-ethylmaleimide in the case of the carnitine exchange and 5 μl of 200 mM phthalonate in the case of the oxoglutarate exchange. In control samples, the inhibitor was added together with the labeled substrate. The assay temperature was 25 °C. In order to remove the external radioactivity, 100 μl of each sample were passed through a Sephadex G-50 column 0.6 × 8 cm. The liposomes eluted with 1.3 ml 50 mM NaCl were collected in 4 ml of scintillation mixture, vortexed and counted. The transport activity was evaluated as the difference between the experimental and the control values from measurements performed after 10 min incubation. In the time-course of the uptake kinetics the measurement at 10 min does not markedly deviate from the linear part of the uptake.

Other methods. Polyacrylamide slab-gel electrophoresis of acetone-precipitated samples was performed in the presence of 0.1% SDS according to Laemmli [17]. A mini gel system was used: gel sizes were 8 × 10 cm × 0.75 mm (thickness). The stacking gel contained 5% acrylamide and the separation gel contained 17.5% acrylamide and an acrylamide-to-bisacrylamide ratio of 150. Staining was performed by the silver nitrate method [18]. Protein was determined by the Lowry method modified for the presence of Triton [19]. All the samples used for protein determination were subjected to acetone precipitation and redissolved in 1% SDS. We have checked that acetone precipitation did not cause any loss of protein. The activity of other transport systems was assayed by the inhibitor stop method [16] as described for each transport system measured [20].

Results and Discussion

Purification of the carnitine carrier

It is known that the activity of the reconstituted carnitine carrier strictly depends on the presence of cardiolipin [21]. Since certain steps in our procedure of purification had to be performed in the absence of cardiolipin, as described below, it was important to verify that no irreversible inactivation of the carrier occurred under these conditions. Thus, the effect of cardiolipin on the carnitine carrier activity has been carefully analyzed. As shown in Table I, the addition of cardiolipin during the solubilization of mitochondria (line B) caused a marked increase in the activity with respect to the control (line A). The same result was obtained when cardiolipin was added only during the chromatography on HTP (line C). Again the same level of activation was obtained when cardiolipin was added only during the reconstitution procedure (line D). These data demonstrate that cardiolipin can be added in any step during isolation and reconstitution of the carnitine carrier to obtain full activity. This means that the inactivation of the carnitine carrier due to the absence of cardiolipin is completely reversible. Furthermore, as

TABLE I

Effect of cardiolipin on the carnitine carrier

The different steps of the purification of the carnitine carrier were performed in the presence (+) or in the absence (−) of cardiolipin, added at a concentration of 2.0 mg/ml. The activity was measured in proteoliposomes reconstituted with the HTP eluate. The proteoliposomes contained 20 mM carnitine and the exchange was started by adding 0.1 mM external [^{3}H]carnitine. The activity of the reconstituted carnitine transport is expressed in μmol/10 min per g protein. The data are from a representative experiment. Similar results were obtained in four different experiments.

	A	B	C	D	E	F
Solubilization	−	+	−	−	+	−
HTP elution	−	−	+	−	+	+
Reconstitution	−	−	−	+	−	+
Specific activity	114	220	244	250	195	230

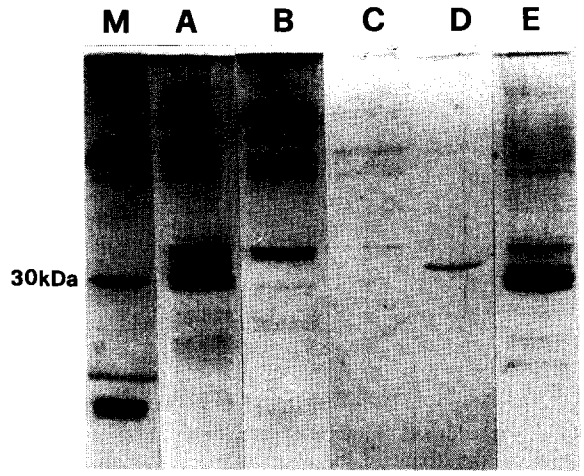

Fig. 1. Purification of the carnitine carrier. SDS-gel electrophoresis of fractions obtained by HTP and celite chromatography. M, marker proteins (bovine serum albumin, carbonic anhydrase and cytochrome *c*); (A) hydroxyapatite eluate (10 μl); (B, C, D) first, second and third fraction of celite eluate, respectively (50 μl in (B) and 300 μl in (C) and (D); (E) residual eluate of the celite column eluted with 1% SDS (50 μl). The numbers reported in the figure represent the specific activities of carnitine/carnitine and oxoglutarate/oxoglutarate exchanges measured in each fraction as described in Materials and Methods. They are expressed as μmol/10 min per g protein.

shown in columns E and F of Table I, there was no additive effect when cardiolipin was added in two different steps, suggesting saturation behaviour. Under our conditions maximum activation was reached with 1.6 mg/ml of cardiolipin, in accordance with previous findings [21].

We previously achieved a partial purification of the carnitine carirer [10]; the first step of the purification procedure described here, i.e., the HTP chromatography, is essentially the same, except that the amount of HTP has been reduced to 1.5 g. This variation resulted in a better yield of the purified carnitine carrier, even though the specific activity found in the HTP eluate (Fig. 1, line A, and Table II) was lower as compared to

TABLE II

Purification of the carnitine carrier

The proteoliposomes were loaded with 20 mM carnitine and the exchange was started by adding 0.1 mM external [^{3}H]carnitine. Other conditions as described in Materials and Methods. The activity of the reconstituted carnitine transport is expressed in μmol/10 min per g protein (specific activity) and μmol/10 min (total activity).

	Protein (mg/ml)	Specific activity	Total activity	Purification (fold)
Extract	8.9	2.7	24.0	-
HTP	0.09	213	19.2	79
Celite	0.0039	2628	10.2	973

the previous paper [10]. In order to purify the carnitine carrier protein further, we have applied the HTP eluate onto various resins already used for other mitochondrial carriers (see Ref. 22 for a review and Refs. 23,24). Among the tested resins (Fractogel, Affi-Gel, Silica-gel 60, celite) only celite was effective for the purification of the carnitine carrier protein in the active form. Fractogel resulted in irreversible binding of the protein, Silica-gel led to the elution of active carnitine carrier but without substantial purification with respect to the HTP column, and the use of Affi-Gel resulted in the elution of two protein bands without carnitine exchange activity.

The previous observation that the carnitine carrier is bound to celite in the absence of cardiolipin [10] suggested the application of the HTP eluate onto a dry celite column without addition of cardiolipin, followed by elution with the same buffer plus cardiolipin. After this procedure the eluate exhibited carnitine exchange activity when reconstituted into liposomes, but two or three bands were still present in this eluate, the mass of which ranged in the 30 kDa region; additionally some bands at higher mass were observed (not shown).

The identification of the carnitine carrier and its purification to homogeneity was achieved only after we observed that salts at high ionic strength on the one hand, and cardiolipin on the other, had differential elution properties on celite. Thus the elution of the celite column loaded with the HTP eluate was performed in three steps: (1) 3% Triton X-100/10 mM Na_2SO_4/10 mM Pipes (pH 7) (Fig. 1, lane B). Under these conditions two main protein bands very close to 35 kDa were eluted along with some higher-mass bands; (2) 3% Triton X-100/5 mM Pipes (pH 7) to eliminate the excess of Na_2SO_4 from the column. This caused the elution of few proteins (Fig. 1 lane C); (3) 1.6 mg/ml cardiolipin in a buffer containing 3% Triton X-100/5 mM Pipes (pH 7). A single band was eluted with a mass of 32.5 kDa (Fig. 1, lane D). Lane E of Fig. 1 shows the residual eluate of the celite column obtained by applying 1% SDS. It should be noted that the samples applied to the gel of Fig. 1 corresponded to 10, 50 and 300 μl for lane A, lane B and lanes C-D, respectively.

Since the molecular mass of the pure band corresponded to that of the purified 2-oxoglutarate carrier from rat liver mitochondria [20], in the experiment of Fig. 1 two specific activities were tested: the [^{3}H]carnitine/carnitine exchange and the 2-[^{14}C]oxoglutarate/oxoglutarate exchange. Both activities were present in the HTP eluate (Fig. 1, lane A). Both were absent from the first two fractions of celite (Fig. 1, lanes B,C) and only the carnitine exchange activity was present in the third fraction of celite eluate (Fig. 1, lane D). From these results we conclude that the carnitine carrier is identical to the 32.5 kDa protein purified by this method, having the same apparent molecular mass as the oxo-

glutarate carrier purified by a different method by HTP chromatography [20]. In addition, the activities of various other anion carriers had been tested in the HTP eluate and in the third celite fraction containing the purified carnitine carrier. It was found that the activities of the phosphate, citrate, dicarboxylate and adenine nucleotide carriers were also present in the HTP eluate, whereas none of these activities was present in the third celite fraction (not shown).

The final purification factor (Table II) of the celite fraction containing the carnitine carrier was 973 with respect to the mitochondrial extract. The total activity recovered with the pure protein was about one-half as compared to that of the extract. The protein yield was 0.04%.

Functional characterization of the reconstituted carnitine carrier

The experiments described in this section have been performed with the celite fraction (Fig. 1, lane D) containing the purified carnitine carrier.

In order to confirm that the activity of the observed [^{3}H]carnitine/carnitine exchange was really due to the purified protein, the following controls were performed: no transport activity was found in liposomes reconstituted without the carnitine carrier protein; carnitine exchange activity was negligible when proteoliposomes reconstituted with the carnitine carrier protein had been incubated with 50 μg/ml of trypsin for 3 h at 25°C. It was verified that no decrease of activity occurred in control proteoliposomes after 3 h of incubation, without trypsin, at 25°C.

It is known from intact mitochondria that the carnitine carrier can catalyze the transport of carnitine and acylcarnitines of various chain lengths by an exchange mechanism [8]. Consistently (Table III) when proteoliposomes were loaded with acylcarnitines of various chain lengths, instead of carnitine, they transported

TABLE III

Dependence of carnitine transport in reconstituted liposomes on internal substrate

The proteoliposomes were loaded with the indicated substrates. Transport was started by the addition of 0.1 mM [^{3}H]carnitine.

Internal substrate (20 mM)	Carnitine transport (μmol/10 min per g protein)
L-Carnitine	2280
L-Acetylcarnitine	2820
L-Octanoylcarnitine	3250
L-Palmitoylcarnitine	2050
– (NaCl)	33
Phosphate	41
Malate	25
Aspartate	55
Citrate	82

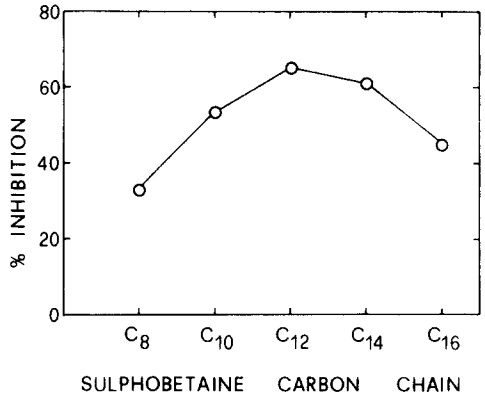

Fig. 2. Dependence of the inhibition of the reconstituted carnitine carrier on the length of the hydrophobic moiety of sulphobetaines. The proteoliposomes were loaded with 20 mM carnitine and the exchange was started by adding 0.1 mM external [^{3}H]carnitine. The various sulphobetaines were added together with the labeled substrate at 0.6 mM concentration. The control value of uninhibited carnitine exchange was 2550 μmol/10 min per g protein.

[^{3}H]carnitine with the same activity. On the other hand, if no substrate was present inside the liposomes, no transport of carnitine occurred. Likewise, if substrates of other carriers were present inside the liposomes, transport of carnitine was also not observed. This gives clear evidence that only carnitine and acylcarnitines can be specifically exchanged with carnitine by the reconstituted protein.

The inhibition of the carnitine transport by sulphobetaines [11] depended on the length of the respective carbon chains, as shown in Fig. 2. We observed a maximum of inhibition with sulphobetaines containing 12–14 carbon atoms in the hydrophobic moiety. This suggests a specific hydrophobic interaction between the carrier and the inhibitor molecule. It should be pointed out that none of the sulphobetaines tested exerted a detergent effect at the concentration used in this experiment.

Table IV shows the effect of various inhibitors on the reconstituted carnitine exchange. As reported for the carrier activity in intact mitochondria [11], the reconstituted carnitine carrier was inhibited by several SH-reagents. At 0.1 mM carnitine the inhibition by *N*-ethylmaleimide and *N*-eosin 5-maleimide was nearly complete at concentrations below 1 mM. Mercurial reagents (mersalyl and *p*-hydroxymercuribenzoate) also strongly inhibited the carrier activity, though less efficiently than the maleimides. These results indicate that reduced SH-groups on the carrier molecule are important for transport activity. Indeed, the presence of a SH-reducing reagent during the purification procedure of the carrier was essential (see Materials and Methods). Pyridoxal phosphate was a poor inhibitor of the carnitine exchange activity, in contrast to the strong inhibitory effect exerted on anion carriers like the oxoglutarate and the glutamate/aspartate carrier [20,25]. Competi-

TABLE IV

Effect of inhibitors on the reconstituted carnitine/carnitine exchange

Proteoliposomes were loaded with 20 mM carnitine and the exchange was started by adding 0.1 mM external [^{3}H]carnitine. The inhibitors were added together with the labeled substrate, except for the SH reagents that were added 1 min before. The control value of uninhibited carnitine exchange was 2130 μmol/10 min per g protein.

Inhibitor	Concentration (mM)	Inhibition (%)
N-Ethylmaleimide	0.5	90
	1.0	98
Eosin 5-maleimide	0.25	91
	0.5	96
Mersalyl	1.0	85
p-Hydroxymercuribenzoate	1.0	83
α-Cyanocinnamate	2.0	49
Pyridoxal phosphate	10	33
Phenylsuccinate	10	0
1,2,3-Benzenetricarboxylate	10	0
Butylmalonate	10	2
Phthalonate	10	4
Bathophenanthroline	5	15
Diazepam	0.15	7
	0.3	25
	0.7	36
Octyl glucoside	0.07% (w/v)	24
	0.2%	58
	0.3%	64

tive inhibitors of other mitochondrial carriers [11], i.e., phenylsuccinate, 1,2,3-benzenetricarboxylate, butylmalonate and phthalonate did not inhibit the carnitine carrier. α-Cyanocynnamate, on the other hand, which inhibits the pyruvate carrier by reacting with its SH-groups [26], diminished the carnitine/carnitine exchange, although at much higher concentrations than those required for inhibition of the transport of pyruvate. 5 mM bathophenanthroline, a powerful inhibitor of the dicarboxylate and the oxoglutarate carrier [27], inhibited the carnitine carrier only 15%. Diazepam has recently been demonstrated to inhibit the carnitine carrier in intact mitochondria [28]. Also with the reconstituted carrier there was a clear effect of diazepam, although the extent of inhibition seemed to be lower in the reconstituted system. Octyl glucoside, a competitive inhibitor of the carnitine palmitoyl transferase [1], also inhibited the carnitine carrier. We have checked that the integrity of the liposomes was not affected by the applied concentrations of octyl glucoside.

All these results are in good agreement with the substrate specificity and inhibitor sensitivity of the carnitine transport system described in intact mitochondria.

Conclusion

The data reported above represent, to our knowledge, the first example of purification to homogeneity of the mitochondrial carnitine carrier. This result should be a useful basis for further characterization of this carrier at a molecular level. In addition, the purification of the carnitine carrier described in this paper answers the question raised in the literature concerning the identity of the acylcarnitine transferases (of cytosol and mitochondria) and the translocase [29]. It is unequivocally demonstrated here that the carnitine carrier is an independent functional and structural entity. It is a single polypeptide of 32.5 kDa. This is different from the mass of the purified acyltransferases from liver, ranging from 60 to 90 kDa [1,29].

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