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Purification of the mitochondrial carnitine carrier by chromatography on hydroxyapatite and celite

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The carnitine carrier from rat liver mitochondria has been extracted with Triton X-100 and partially purified by chromatography on hydroxyapatite and celite. During purification the activity of the carrier was monitored by functional reconstitution into liposomes. The purified fraction is 250-fold enriched with respect to the *N*-ethylmaleimide-sensitive carnitine/carnitine transport activity. The substrate specificity and the inhibitor sensitivity of carnitine transport in liposomes resemble closely those described for the transport of carnitine in mitochondria.

Carnitine carrier; Carnitine transport; Liposome; Mitochondria; (Rat liver)

1. INTRODUCTION

The inner mitochondrial membrane contains a specific carrier system for the transport of carnitine (for a review see [1]). Under physiological conditions this system catalyzes the exchange between external acylcarnitine and matrix carnitine, which is an important step in the mitochondrial oxidation of fatty acids. The properties of the carnitine carrier have been extensively investigated in intact mitochondria [2–7]. More recently, Noël et al. [8,9] have reconstituted the transport of carnitine in liposomes using total mitochondrial extract. However, purification of the transport system has not yet been achieved.

In this report we describe a partial purification of the carnitine carrier from rat liver mitochondria. The carrier is about 250-fold enriched and, when reconstituted into phospholipid vesicles, closely resembles the properties of the carnitine transport system as characterized in intact mitochondria.

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2. MATERIALS AND METHODS

Hydroxyapatite (Bio-Gel HTP) was purchased from Bio-Rad, Celite 535 from Roth, Amberlite XAD-2 from Fluka, L-[^{3}H]carnitine from Amersham, phospholipids (phosphatidylcholine from fresh turkey egg yolk), cardiolipin, Pipes (1,4-piperazinediethanesulfonic acid), Triton X-100, L-carnitine, DL-octanoylcarnitine, *N*-octyl- and *N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate from Calbiochem. All other reagents were of analytical grade.

Rat liver mitochondria prepared by standard procedures were solubilized in 2.5% Triton X-100 (w/v)/50 mM NaCl/1 mM DTE/10 mM Pipes, pH 7.0 (16–18 mg protein/ml), and centrifuged at 100000 × *g* for 30 min at 0°C. 0.6 ml of the supernatant (extract) were applied to a dry hydroxyapatite (2 g) column and eluted with 1% Triton X-100/20 mM Na_2SO_4/1 mM DTE/10 mM Pipes, pH 7.0, at 0.05 ml/min. The first 0.5 ml of the eluate, supplemented with cardiolipin (2 mg), were applied to a dry celite (0.25 g) column and eluted with the same hydroxyapatite buffer plus cardiolipin (4 mg/ml). Protein eluates were reconstituted by the amberlite method as described in [10,11], except that the reconstitution mixture consisted of 200 μl of the hydroxyapatite or celite eluate or 50 μl of the extract, 15 μl cardiolipin (40 mg/ml), 60 μl of 10% Triton X-100, 80 μl liposomes (10% phospholipids), 20 mM carnitine, 50 mM Na_2HPO_4, pH 7.0, in a final volume of 550 μl. The external substrate was removed by chromatography of the liposomes on Sephadex G-75 pre-equilibrated with 20 mM NaCl/50 mM Na_2HPO_4. Transport measurements were performed by the inhibitor stop method [12] by adding 0.1 mM [^{3}H]carnitine to the proteoliposomes at

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time zero and using 2 mM *N*-ethylmaleimide as stopping reagent. In control samples, the inhibitor was added together with the labelled substrate. The external radioactivity was removed by applying 50 μl of the samples on Sephadex G-50 columns (5.5 × 0.6 cm). The liposomes were eluted within 1.1 ml sample using 50 mM NaCl. The eluted liposomes were collected in the scintillation mixture (maxifluor, Baker) and counted. The transport activity was calculated by subtracting the control from the experimental values. Polyacrylamide slab gel electrophoresis was performed in the presence of 0.1% SDS according to Laemmli [13]. Protein was determined by the Lowry method modified for the presence of Triton [14].

3. RESULTS AND DISCUSSION

3.1. *Isolation and partial purification*

In order to isolate the carnitine carrier, the Triton X-100 extract of liver mitochondria was separated by various techniques and the different fractions were analyzed for carnitine transport activity and the polypeptide pattern in SDS-gel electrophoresis. An efficient purification was obtained by applying the crude extract of liver mitochondria to hydroxyapatite (table 1). The carnitine carrier passed through hydroxyapatite whereas most of the other proteins were retained under these conditions. Critical factors for optimal purification were found to be the rate of elution and the hydroxyapatite/protein ratio. The activity of the carnitine carrier was retained by the column when the rate of elution was below 0.02 ml/min and the hydroxyapatite/protein ratio (w/w) higher than 200. Under the conditions described in section 2, chromatography on hydroxyapatite increased the specific activity of the reconstituted carnitine/carnitine exchange 94-fold. The total activity in the eluate after hydroxyapatite accounted for 79% of that applied to the column.

For further purification, the hydroxyapatite pass-through had to be supplemented with cardiolipin and subjected to chromatography on celite (table 1). By this purification step the specific activity of the reconstituted carnitine transport was further increased 2.7-fold. It should be stressed that in the absence of cardiolipin the carnitine carrier was retained by celite. The entire purification procedure including addition of cardiolipin increased the specific activity of reconstituted carnitine transport 252-fold as compared to that of the mitochondrial extract. Approx. 48% of the total transport activity was recovered with a protein yield of 0.2%. The SDS-gel electrophoresis of the final eluate showed 4–5 protein bands (fig.1, lane D), the most prominent of which at M_r 30000 and 35500 correspond to the ADP/ATP carrier and porin, respectively. The 35500 Da band was the only protein eluted from celite in the absence of cardiolipin (lane C) and it has been identified with porin both functionally and immunologically (not shown).

Table 1

Purification of the carnitine carrier by chromatography on hydroxyapatite and celite

	Protein (mg/ml)	Specific activity	Total activity	Purification (fold)
Triton X-100 mitochondrial extract	9.4	2.5	23.5	–
Hydroxyapatite	0.076	235	17.9	94
Celite	0.018	630	11.3	252

Conditions as described in section 2. The activity of the reconstituted carnitine/carnitine exchange is expressed as μmol/10 min per g protein (specific activity) and μmol/10 min (total activity)

3.2. *Properties of the reconstituted carnitine carrier*

The substrate specificity of the partially purified carnitine carrier was investigated by changing the substrate entrapped in the liposomes as well as by studying the effect of externally added substrates on the [^{3}H]carnitine/carnitine exchange. The data illustrated in fig.2A show that [^{3}H]carnitine exchanges not only with internal carnitine but also with DL-octanoylcarnitine. The uptake of labelled carnitine was negligible when the proteoliposomes were not loaded with carnitine, as found in mitochondria [5]. Virtually no uptake occurred without incorporation of the purified fraction into the liposomes or when the proteoliposomes contained substrates of other mitochondrial carriers like citrate, ADP, phosphate and oxoglutarate (not shown). Among the externally added substrates, DL-octanoylcarnitine strongly inhibited the carnitine/carnitine exchange (fig.2B). In contrast, ADP, citrate, phosphate, oxoglutarate, malate and aspartate had very little effect (not shown). The inhibitor sensitivity of the purified carnitine transport protein is shown in fig.2B. As in mitochondria [4,7], the reconstituted carnitine exchange was inhibited by mersalyl, *N*-octyl-*N,N*-

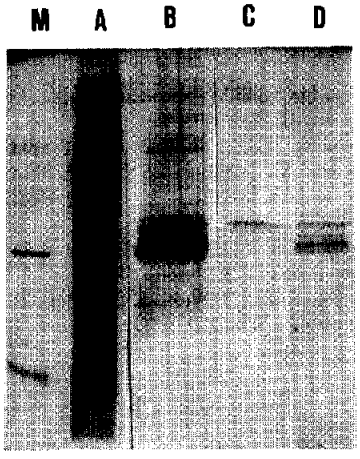

Fig.1. SDS-gel electrophoresis of fractions obtained during the purification of the carnitine carrier. (M) Marker proteins (bovine serum albumin, carbonic anhydrase and cytochrome *c*); (A) mitochondrial extract; (B) hydroxyapatite eluate; (C) celite eluate without added cardiolipin; (D) celite eluate with added cardiolipin.

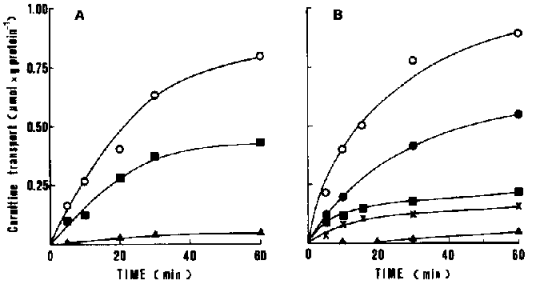

Fig.2. Substrate specificity and inhibitor sensitivity of the carnitine transport protein in reconstituted liposomes. (A) 0.1 mM [^{3}H]carnitine was added at time zero to proteoliposomes with 20 mM carnitine (○) or 40 mM DL-octanoylcarnitine (■) as internal substrate, or to proteoliposomes without internal substrate (▲). (B) Proteoliposomes were loaded with 20 mM carnitine and the exchange was started with 0.1 mM external [^{3}H]carnitine (○). The inhibitors 1.7 mM *N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate (●), 5 mM DL-octanoylcarnitine (■), 8 mM *N*-octyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate (×) were added simultaneously with [^{3}H]carnitine. Mersalyl (▲) was added 1 min before the labelled substrate at a concentration of 1 mM.

dimethyl-3-ammonio-1-propanesulfonate and *N*-dodecyl-*N,N*-dimethyl-3-ammonio-1-propanesulfonate, besides *N*-ethylmaleimide used in the present paper as stop inhibitor of the carnitine transport. We have tested that the above mentioned sulfobetaines do not exert detergent effect at the concentrations used in this experiment. In contrast, known inhibitors of other mitochondrial carriers (carboxyatractyloside, 1,2,3-benzenetricarboxylate, butylmalonate and phthalonate) had no significant effect.

The time course of the carnitine/carnitine exchange, as reported in fig.2, can be analyzed kinetically [15]. By plotting the logarithm of the ratio $\text{carnitine}_{max}/(\text{carnitine}_{max} - \text{carnitine}_t)$ against time, a straight line was obtained, which demonstrates that the reconstituted carnitine/carnitine exchange follows a first-order kinetics, as found for other mitochondrial carrier-mediated transports [16,17]. The first-order rate constant, k, was 0.04 min^{-1}.

In conclusion, it has been shown that a protein fraction with a 250-fold enriched specific transport activity contains the functionally active carnitine carrier. The reconstituted protein exhibits the same substrate specificity and inhibitor sensitivity of the transport system in mitochondria. Since the purity of the reconstituted protein fraction is still unknown, the specific activity of carnitine exchange obtained in proteoliposomes cannot be correlated quantitatively to the original mitochondrial activity. However, the rate of reconstituted carnitine/carnitine exchange is in the same order of magnitude as the values found for other reconstituted mitochondrial metabolite carriers [18,19] and definitely lower than the rate of the reconstituted phosphate carrier [20] which also has a higher specific activity in mitochondria.

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