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CARNITINE TRANSPORT IN SUBMITOCHONDRIAL PARTICLES AND RECONSTITUTED PROTEOLIPOSOMES*

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Summary: Influx and efflux measurements of carnitine with submitochondrial particles lead to the conclusion that carnitine can cross the inner mitochondrial membrane by either facilitated diffusion or more rapidly by a carnitine-carnitine exchange. Both, the facilitated diffusion and the exchange are inhibited by N-ethylmaleimide or mersalyl at low concentrations. Reconstituted particles prepared from liposomes and either submitochondrial particles or an octyl β-glucoside-solubilized preparation were active in catalyzing carnitine-carnitine exchange.

Carnitine participates in the transfer of long chain fatty acids across the inner mitochondrial membrane (1). Pande (2,3) as well as Ramsay and Tubbs (4) demonstrated that carnitine and acylcarnitines enter the matrix space of mitochondria by a 1:1 exchange with internal carnitine. Since the availability of a simple system capable of catalyzing carnitine exchange would greatly aid further investigations of fatty acid translocation across the inner mitochondrial membrane, we have studied carnitine uptake by submitochondrial particles and reconstituted proteoliposomes.

EXPERIMENTAL PROCEDURES

Materials - DL-Carnitine hydrochloride was purchased from Sigma Chemical Co., D-carnitine hydrochloride was bought from ICN

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Abbreviations: MOPS, 3(N-morpholinolpropanesulfonic acid; DTT, dithiothreitol; NEM, N-ethylmaleimide; EDTA, ethylenediamine tetraacetic acid.

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Pharmaceuticals, Inc., and L-carnitine hydrochloride was a gift of Dr. K. Brendel, University of Arizona, College of Medicine. Soybean phospholipids were obtained from Associated Concentrates, Woodside, N.Y. DL-[Methyl-^{14}C]carnitine hydrochloride was purchased from Amersham/Searle Co. L-[Methyl-^{14}C]carnitine was prepared from the racemic mixture by conversion of L-carnitine to its palmitoyl derivative in the presence of palmitoyl-CoA, 5,5'-dithiobis(2-nitrobenzoic acid) and submitochondrial particles. The resulting palmitoyl-L-carnitine was extracted from the reaction mixture with n-butanol and hydrolyzed with 0.1 N KOH to yield L-[methyl-^{14}C]carnitine. Bovine heart mitochondria were isolated as described by Blair (5). In order to deplete mitochondria of carnitine, they were shortly sonicated (15 sec with a Branson sonifier, 1/2" tip, 60-70 W output) and separated from the suspension medium by centrifugation at 27,000 xg for 20 min. The pellet was washed twice with 0.25 M sucrose. Submitochondrial particles were prepared by sonication of the presonicated mitochondria following the procedure of Hansen and Smith (6). Submitochondrial particles prepared in the presence (carnitine-loaded vesicles) or absence (control vesicles) of added carnitine were finally suspended in 10 mM MOPS (pH 7.5)-0.25 M sucrose. Immediately before use, they were rapidly passed through Sephadex G-50 (fine) equilibrated with 10 mM MOPS (pH 7.5)-0.25 M sucrose to remove residual amounts of external carnitine, following the procedure of Penefsky (7).

Reconstitution and Assay of Carnitine Transporter - A suspension of 4% soybean phospholipids in 10 mM MOPS (pH 7.5)-0.1 M KCl was sonicated to clarity under N_2 in a bath-type sonicator (Sonicator Laboratory Supply Co., Hicksville, N.Y.) and combined with an equal volume of 10 mM MOPS (pH 7.5)-0.1 M KCl containing either 45 mM carnitine (carnitine-loaded vesicles) or no carnitine (control vesicles) and 0.5-2 mg/ml of submitochondrial particles or an octyl β-glycoside-solubilized preparation. The mixture was frozen in liquid N_2, allowed to thaw at 20°, sonicated for 1 min under N_2 in a bath-type sonicator and rapidly filtered through Sephadex G-50 (fine) equilibrated with 10 mM MOPS (pH 7.5)-0.1 M KCl as described by Penefsky (7) to remove external carnitine. These proteoliposomes (0.1 ml) were assayed for carnitine uptake by incubation with 5 µl of 2 mM DL-[^{14}C]carnitine (0.5 µCi) at 20°. The assays were terminated by separating the vesicles of 75 µl samples from the suspending medium by rapid filtration through Sephadex G-50 (fine) as described by Penefsky (7). The filtered proteoliposomes were suspended in 1 ml of water, dissolved in 10 ml of Liquiscint (National Diagnostics) and analyzed for radioactivity in a Beckman scintillation counter. The difference in carnitine uptake between carnitine-loaded and control vesicles is referred to as carnitine exchange.

Submitochondrial particles (2.3 mg/ml) were solubilized by incubation in 45 mM KP_i (pH 7.5) containing 5 mM EDTA, 2 mM DTT, 10 mM DL-carnitine, 5% glycerol and 35 mM octyl β-glucoside for 30 min at 0°. After centrifugation at 140,000 x g for 60 min the supernatant was concentrated 10-fold in an Amicon concentrator (YM-10 membrane) and brought to 55% saturation with a concentrated solution of $(NH_4)_2SO_4$ containing 10 mM Tris-sulfate (pH 7.5)-0.5 mM EDTA. The $(NH_4)_2SO_4$-precipitate was collected by centrifugation and dissolved in 10 mM MOPS (pH 7.5)-0.1 M KCl to give a protein concentration of 5.5 mg/ml.

RESULTS AND DISCUSSION

Carnitine Uptake by Submitochondrial Particles - Carnitine transport across the inner mitochondrial membrane was studied by measuring the influx of L-[^{14}C]carnitine into submitochondrial particles. The rapid uptake of carnitine by submitochondrial particles loaded with either L-carnitine or D-carnitine as compared to that by control vesicles, which did not contain carnitine (see Fig. 1A), demonstrates the operation of a carnitine exchange system similar to that observed in whole mitochondria (2-4). The initial rate of exchange of L-carnitine vs. L-carnitine was slightly faster than that of L-carnitine vs. D-carnitine at an internal carnitine concentration of 1 mM (see Fig. 1A). At 3 mM no rate difference was observed (data not shown). This finding suggests that the exchange system operates at the same maximal rate with either L-carnitine or D-carnitine but has a slightly higher affinity for L-carnitine than for the D-enantiomer. Higher rates of L-carnitine vs. L-carnitine exchange as compared to the L-carnitine vs. D-carnitine exchange have also been observed with whole mitochondria (3,4). Since carnitine uptake by control vesicles, which did not contain any internal carnitine, was inhibited by N-ethylmaleimide or mersalyl at low concentrations, it appears that carnitine can cross the inner mitochondrial membrane by facilitated diffusion. In order to provide further proof for this point, the efflux of carnitine from submitochondrial particles preloaded with DL-[^{14}C]carnitine was determined. As shown in Fig. 1B, carnitine was rapidly released from these vesicles in the absence of external carnitine although the presence of external carnitine accelerated this process presumably due to the higher rate of exchange as compared to the rate of facilitated diffusion. The rate of carnitine efflux was greatly reduced at

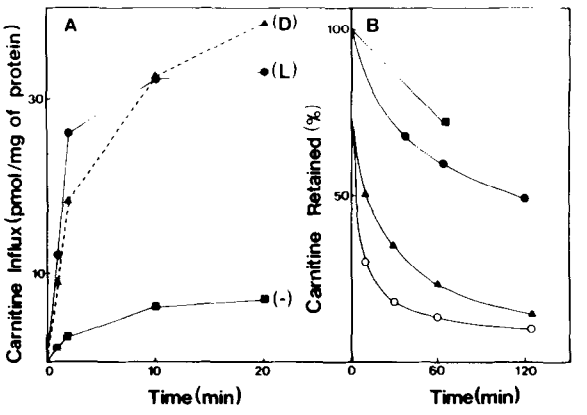

Fig. 1. Carnitine uptake and efflux measurements with submitochondrial particles. A. Carnitine uptake: To 0.15 ml of submitochondrial particles (0.75 mg of protein) in 10 mM MOPS (pH 7.5)-0.25 M sucrose were added 5 µl of 0.6 mM L-[^{14}C]carnitine (0.15 µCi). After incubating the mixture at 10° for the indicated time periods vesicles were separated from the suspension medium and counted for radioactivity as described for proteoliposomes under "Experimental Procedures". All values were corrected for counts associated with NEM-treated particles. Submitochondrial particles loaded with 1 mM L-carnitine (●), 1 mM D-carnitine (▲) and no carnitine (■) were used. B. Carnitine efflux: 1.5 ml of submitochondrial particles loaded with 2 mM DL-carnitine and suspended in 10 mM MOPS (pH 7.5)-0.25 M sucrose were incubated with 30 µl of 2 mM DL-[^{14}C]-carnitine (3 µCi) for 30 min at 10°. The suspension medium was replaced with 10 mM MOPS (pH 7.5)-0.25 M sucrose by filtration through Sephadex G-50 (fine) by the procedure of Penefsky (7). The resulting suspension of submitochondrial particles (0.15 ml containing 0.63 mg of protein and 2,200 cpm) was incubated as follows: o, at 10°, with 2.4 mM external DL-carnitine; ▲, 10°, no external carnitine; ●, 0°, no external carnitine; ■, 10°, no external carnitine, plus 0.16 mM NEM. The separation of the vesicles from the suspension medium was identical to that described for proteoliposomes under "Experimental Procedures".

0°C and was inhibited by N-ethylmaleimide (see Fig. 1B). The observations presented above suggest that carnitine can cross the inner mitochondrial membrane by facilitated diffusion which proceeds more slowly than the carnitine-carnitine exchange.

As shown in Table I, the carnitine exchange is sensitive towards sulfhydryl modifying agents as for example N-ethylmaleimide and mersalyl. Since the same observation has been made with whole mitochondria (3), and since N-ethylmaleimide does not penetrate into submitochondrial particles (8), sensitive sulf-

Table I

Inhibition of Carnitine Exchange in Submitochondrial Particles

Inhibitor	Conc. μM	Carnitine Exch. pmol/10 min/mg	Inhibition %
None		44	
NEM	9.5	27	39
NEM	32	0	100
Mersalyl	9.5	28	36
Mersalyl	95	1.3	97

Submitochondrial particles were incubated for 10 min at 10° with either NEM or mersalyl and were then assayed for carnitine exchange as described in the legend to Fig. 1

hydryl group(s) essential for carnitine exchange seem to face both sides of the inner mitochondrial membrane. Submitochondrial particles treated with low levels of N-ethylmaleimide (6 nmol/mg) were inactive with respect to carnitine transport but were unimpaired in carnitine palmitoyltransferase activity. This observation suggests that the carnitine palmitoyltransferase is not directly involved in the transport of carnitine across the inner mitochondrial membrane.

Reconstitution of Vesicles Active in Carnitine Exchange - Proteoliposomes prepared from submitochondrial particles and liposomes by freeze-thawing followed by sonication catalyzed carnitine exchange (see Fig. 2) in contrast to pure liposomes which were inactive (see Table II). The carnitine exchange in reconstituted vesicles was inhibited by N-ethylmaleimide although the concentration of inhibitor required to obtain nearly complete inhibition was higher than that necessary to inhibit the exchange in submitochondrial particles (see Tables I and II). When submitochondrial particles were first treated with N-ethylmaleimide and then incorporated into liposomes, the carnitine exchange was strongly inhibited (see Table II). The rate of carnitine exchange was linear with respect to protein over the concentration

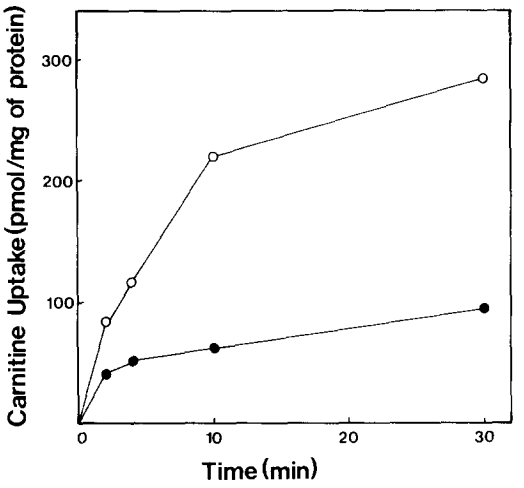

Fig. 2 Time course of carnitine uptake by proteoliposomes reconstituted from lipsomes and submitochondrial particles. The preparation of the vesicles and the assay conditions are described under "Experimental Procedures". o, vesicles loaded with either 45 mM L-carnitine or D-carnitine; ●, control vesicles without internal carnitine.

Table II

Carnitine Exchange in Reconstituted Vesicles

Vesicles[a]	Changes	Carnitine Uptake	Carnitine Exch.
		pmol/10 min/2 mg of soybean phospholipids	
(DL)	None	22.4	15 (100%)
(-)	"	7.3	
(DL)	Protein deleted	4.4	0
(-)	"	4.4	
(DL)	0.93 mM NEM added	5.2	0.9 (6%)
(-)	"	4.3	
(DL)	NEM-treated submitochondrial particles[b]	8.6	3.5 (23%)
(-)		5.1	

The preparation of reconstituted vesicles and the assay conditions are presented under "Experimental Procedures". Vesicles containing 2 mg of soybean phospholipids and 90 µg of submitochondrial protein were used per assay except when pure liposomes were assayed.

[a](DL), vesicles loaded with DL-carnitine; (-), vesicles prepared in the absence of carnitine.

[b]Submitochondrial particles pretreated with 0.33 mM NEM were used for the preparation of proteoliposomes.

range used in this study (data not shown) and increased from 15 to 360 pmol/min/mg of protein when the external carnitine concentration was raised from the usual 95 μM to 4.4 mM.

Proteoliposomes were also prepared by incorporation into liposomes of soluble proteins extracted with octyl β-glucoside from submitochondrial particles as described under "Experimental Procedures." The specific activity of the resulting vesicles was 30 pmol/min/mg of protein as compared to 15 pmol/min/mg of protein obtained with submitochondrial particles. Approximately 50% of the carnitine exchange activity was recovered in the reconstituted proteoliposomes. When other detergents, as for example deoxycholate and Triton X-100, were used, inactive vesicles were obtained. The successful reconstitution into liposomes of soluble proteins extracted with octyl β-glucoside from submitochondrial particles provides a valuable tool for further investigations of the poorly understood translocation of fatty acids across membranes.

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