THE MECHANISM OF FATTY ACID UPTAKE BY HEART MITOCHONDRIA: AN ACYLCARNITINE–CARNITINE EXCHANGE

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1. Introduction

Is is now accepted that carnitine is involved in the transport of fatty acyl groups into mitochondria [1,2] and is essential for the oxidation of long-chain fatty acids. According to the original proposals carnitine and its esters, unlike CoA, could penetrate the mitochondrial inner membrane; however, experiments using rat liver mitochondria showed that carnitine could only penetrate the same mitochondrial space as sucrose [3,4] and that swelling did not occur when mitochondria were suspended in an iso-osmotic carnitine solution [5]. These findings, together with the fact that isolated rat liver mitochondria contain less than 0.1 nmol of L-carnitine per mg of protein, have led to the hypothesis that the 'inner' carnitine acyltransferase is vectorial [3] and catalyses a direct acyl transfer between external carnitine and matrix CoA.

We have briefly reported [6] that ox heart mitochondria contain 2–4 nmol of L-carnitine per mg of protein. The results in the present paper demonstrate that this exchanges in a 1:1 manner with external carnitine. We suggest that a carnitine:acylcarnitine exchange is the mechanism of fatty acyl transport across the mitochondrial inner membrane.

2. Materials and methods

2.1. *Preparation of mitochondria*

Fresh ox heart was blended for 30 sec with 3 vol of cold 0.3 M sucrose containing 10 mM 3-(*N*-morpholino) propanesulphonic acid and 2 mM EGTA* adjusted to pH 7.2 with KOH. All subsequent operations were at 0–4°C. The homogenate was centrifuged at 500 *g* for 10 min and the supernatant filtered through muslin and centrifuged for 10 min at 15 000 *g*. The mitochondria were washed twice and suspended in the sucrose medium (containing 1 mM EGTA). No light mitochondria were seen; red cells were discarded at each resuspension.

Protein concentrations were measured by the Lowry method [7] using bovine serum albumin standards in the sucrose medium.

2.2. *L-carnitine assay*

For measurement of free L-carnitine mitochondria were deproteinized with 3% $HClO_4$ and the extracts neutralized with 2 M KOH containing 0.1 M Tris. Samples for measurement of total L-carnitine were incubated in 0.1 M KOH at 70°C for 1 hr and deproteinized as before.

Carnitine samples (2–20 nmol) were mixed with 2 ml of 100 mM potassium phosphate, pH 7.2, containing 1 mM EGTA, 40 μM acetyl-CoA and 125 μM 4,4′-dithiobispyridine. When reduction of dithiobispyridine (followed at 324 nm [8]) ceased, carnitine acetyltransferase (about 15 μg) was added; the concentration of carnitine was calculated assuming an extinction coefficient of 19.8×10^3 M^{-1} cm^{-1} for 4-thiopyridone [8]. Dithiobispyridine is superior to DTNB [9] because it does not inactivate carnitine acetyltransferase.

2.3. *Radioactive carnitine*

DL-[Me-^{14}C] carnitine (The Radiochemical Centre, Amersham) was diluted with unlabelled DL-carnitine to a specific radioactivity of 5 μCi/μmol. Enzymic

* *Abbreviation:* EGTA, ethanedioxybis (ethylamine) tetra-acetic acid.

acetylation of the L-isomer was carried out at 30°C in a system containing 10 μmol DL-[^{14}C]carnitine, 10 μmol acetyl-CoA, 10 μmol 4,4′-dithiobispyridine and 250 μg carnitine acetyltransferase in 20 ml of 5 mM Tris–HCl, pH 7.5. After 2 hr the mixture was acidified with $HClO_4$, concentrated to about 5 ml by rotary evaporation and applied to a column (57 × 1.7 cm) of Zerolit 225 sulphonate resin previously equilibrated with 0.15 M formic acid adjusted to pH 4.5 with trimethylamine. Acetyl-L-[^{14}C]carnitine was eluted before D-[^{14}C]carnitine with this buffer, which was removed from the radioactive fractions by rotary evaporation. Acetyl-L-[^{14}C]carnitine was deacetylated by incubation at 30°C in 2 M NH_4OH for 1 hr and dried in vacuo. DL-[Me-^{3}H]carnitine was resolved in the same way.

Radioactivity was measured by counting aqueous samples (0.25 ml) mixed with 3 ml of toluene: Triton X-100 (3:1) containing 0.5% (w/v) 2,5-diphenyloxazole.

2.4. *Labelling of mitochondrial carnitine by exchange*

Mitochondria (20 mg protein per ml) were incubated for 1 hr at 10°C with 12 μM L-[^{14}C]carnitine (3.45 μCi/μmol), chilled to 0°C, centrifuged for 10 min at 25 000 *g* and washed twice in sucrose medium.

The radioactivity was 'chased' form the mitochondria by exchange with external solutes as described in the legends.

3. Results

3.1. *Endogenous L-carnitine of mitochondria*

Ox heart mitochondria were found to contain 2.85 ± 0.55 nmol of total L-carnitine per mg protein (mean ± S.D. of 30 observations). This amount remained constant after 6 additional washes and decreased by only about 25% after 24 hr at 0°C.

Mithochondria isolated from 120 rat hearts contained 1.31 nmol L-carnitine per mg protein.

Fig.1 shows that most of the carnitine in freshly prepared mitochondria is esterified and that the proportion which is free increases during storage.

3.2. *Stoichiometry of exchange of mitochondrial for external carnitine*

Table 1 (Expt. 1) shows that external L-[^{14}C]carni-

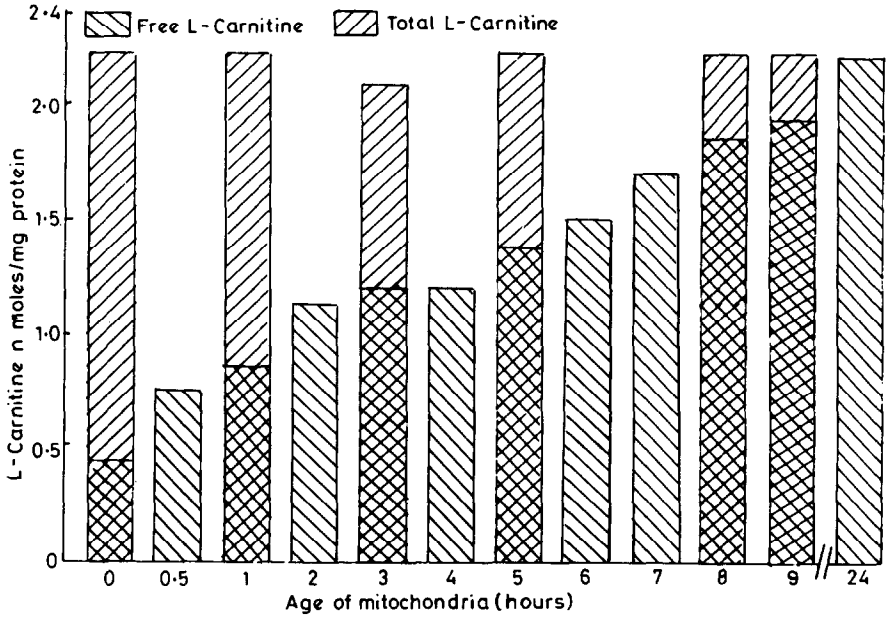

Fig.1. Change in amounts of L-carnitine and acyl-L-carnitine in stored ox heart mitochondria. A mitochondrial suspension (20 mg protein/ml) was kept at 0°C and samples assayed for free and total carnitine (see Materials and methods).

Table 1
Stoichiometry of exchange of mitochondrial for external carnitine

Experiment	Mitochondrial L-carnitine (nmol/mg protein)	L-carnitine exported (nmol/mg protein)	Carnitine imported (nmol/mg protein)	Ratio $\frac{\text{Carnitine exported}}{\text{Carnitine imported}}$	
(1) External L-[^{14}C]-carnitine	1.67	1.22	1.23	0.99	
Control	1.66				
(2) External D-[^{14}C]-carnitine	1.69	0.88	0.86	1.02	
Control	2.57				
(3) Double-labelling					
(a) [^{14}C] internal, [^{3}H] external.		0.245	0.287	0.85	
		0.567	0.510	1.11	1.06
		0.601	0.604	1.00	± 0.17 (S.D.)
		0.777	0.615	1.26	
(b) [^{3}H] internal, [^{14}C] external.		0.330	0.305	1.08	
		0.570	0.467	1.22	1.15
		0.716	0.583	1.23	± 0.09 (S.D.)
		0.717	0.600	1.06	

Expt. (1) Mitochondria were incubated with 46.5 μM L-[^{14}C]carnitine (0.1 μCi/μmol) for 30 min at 10°C and washed twice; a control sample was incubated without carnitine. Carnitine contents and incorporated radioactivity were measured as described in the text. Expt. (2) was as Expt. (1) except that 200 μM D-[^{14}C]carnitine (0.45 μCi/μmol) was used. In Expt. (3) mitochondria were labelled as in Expt. (1), but incubation was for 1 hr with 12 μM L-[^{14}C]carnitine (3.45 μCi/μmol) or 2 μM L-[^{3}H]carnitine (90 μCi/μmol). The labelled mitochondria were then treated in the same way with the other isotope for various times (22–108 min). Finally the [^{14}C] and [^{3}H] contents were measured.

tine exchanges with endogenous L-carnitine in a ratio of 1:1, so that the mitochondrial carnitine content remains unchanged. In Expt. 2 the L-carnitine decreases by an amount equal to the D-[^{14}C]carnitine imported. The 1:1 ratio of exchange is confirmed by Expt. 3, in which both internal and external pools of L-carnitine were radioactively labelled.

3.3. *Specificity of exchange*

Fig.2a shows the rapid displacement of internal L-[^{14}C]carnitine by external L-carnitine, acetyl-L-carnitine and deoxycarnitine (4-trimethylaminobutyrate); D-carnitine exchanges more slowly. 2-Trimethylaminoacetate (betaine) and 6-trimethylaminohexanoate were not substrates. Fig.2b shows that, at low concentrations, acyl derivatives of L-carnitine displace internal carnitine much more rapidly than does carnitine itself. Ohter experiments (not reported) have shown that carnitine: carnitine exchange also occurs with rat heart mitochondria and with submitochondrial particles prepared by sonication of ox heart mitochondria in the presence of L-carnitine.

4. Discussion

The amount of mitochondrial carnitine represents only about 10% of the total heart carnitine. However, assuming that it is distributed in a matrix volume of 2 μl per mg of mitochondrial protein [10], the internal concentration may be 1–2 mM, similar to that in the cytosol [11].

The results suggest that fatty acyl transport into heart mitochondria may proceed as shown in fig.3, in which an acylcarnitine: carnitine exchange step occurs between the two carnitine acyltransferase reactions. This scheme avoids the need to postulate vectorial properties for the 'inner' carnitine acyltrans-

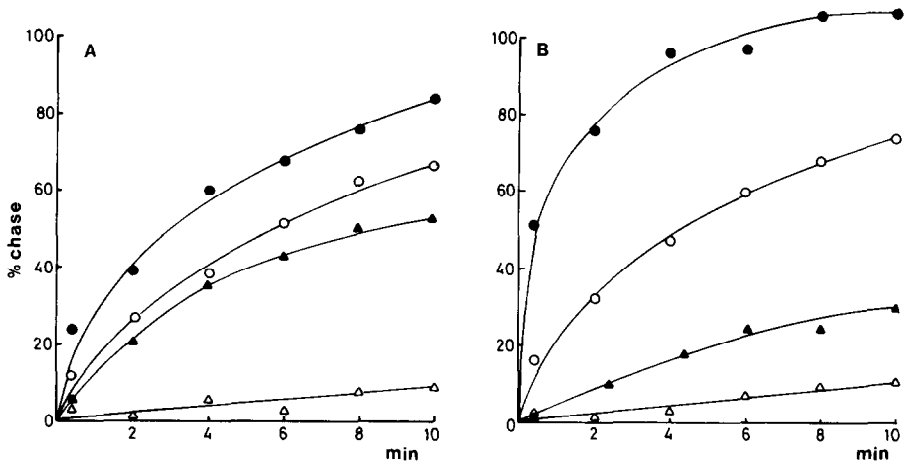

Fig.2. Export of mitochondrial L-[^{14}C]carnitine by exchange with external carnitine derivatives. The intramitochondrial carnitine was labelled as described under Methods. All results are corrected for [^{14}C]carnitine found in control supernatants. (a) Mitochondria (20 mg protein/ml) were incubated at 10°C with 1 mM substrates (●——●) acetyl-L-carnitine; (○——○) L-carnitine; (▲——▲) deoxycarnitine; (△——△) D-carnitine). Samples (250 μl) were centrifuged for 1 min in a Quickfit Instrumentation Micro-centrifuge and 100 μl of the supernatant counted. Results are expressed as percentages of the maximum theoretical 'chase' calculated from the internal and external carnitine concentrations. (b) As in (a) except that bovine serum albumin (5 mg/ml; free of fatty acids), antimycin A (2 μg/ml) and rotenone (2 μg/ml) were present in the incubation. (●——●) 50 μM Lauroyl-L-carnitine; (○——○)200 μM octanoyl-L-carnitine; (▲——▲) 200 μM L-carnitine; (△——△) 50 μM L-carnitine.

ferases [3]. The relationship of the acyltransferases to the exchange system is not clear, although acyl transfer reactions are precluded by the efficacy of D-carnitine and deoxycarnitine. (These substances do however bind to the enzymes [3,12,13]).

It seems likely that the observed rates of exchange are adequate to account for the utilization of acyl-carnitine derivatives by mitochondria. The ability of long-chain derivatives to exchange rapidly at concentrations much lower than those at which carnitine itself is effective would favour the physiological uptake of fatty acyl groups by mitochondria.

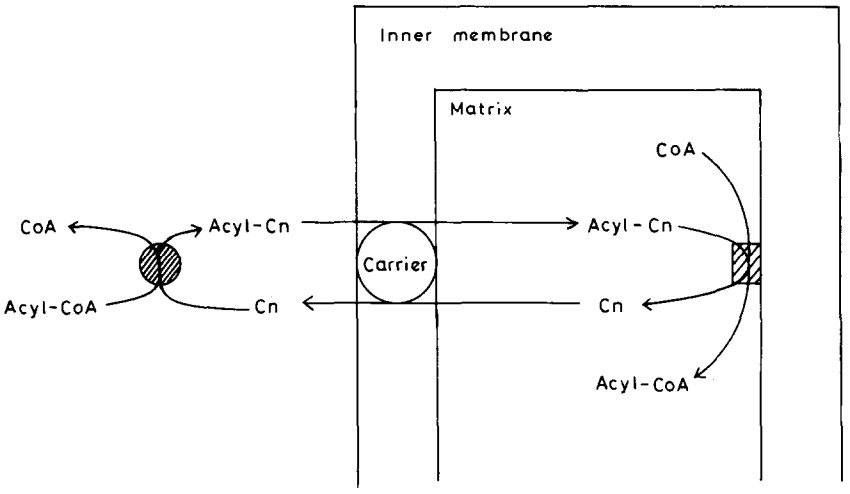

Fig.3. Postulated acylcarnitine-carnitine exchange carrier in the mitochondrial inner membrane.

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