Aggregation of submitochondrial particles by heparin and its application to the study of carnitine transport

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A novel technique for the separation of submitochondrial particles from the external medium, an essential procedure in transport studies, was devised. Very low concentrations of heparin (5–10 $\mu\text{g/ml}$) aggregate the particles and permit their rapid sedimentation in a micro-centrifuge. The transfer of activated fatty acids into mitochondria for oxidation depends on the exchange of matrix carnitine for external fatty-acylcarnitine. To study the matrix face of the carnitine/acylcarnitine translocase, inverted submitochondrial particles were prepared and loaded with L-[^{14}C]carnitine. As found in intact mitochondria, the K_m value for L-carnitine was 8 mM, that for palmitoyl-L-carnitine was two orders of magnitude lower, and 11-trimethylaminoundecanoyl-DL-carnitine was a competitive inhibitor. The properties of the carrier exposed to the outer and to the matrix sides of the mitochondrial inner membrane are thus similar.

INTRODUCTION

Carnitine and its fatty acid esters are transported through the inner membrane of heart mitochondria by a specific exchange carrier (Ramsay & Tubbs, 1975, 1976; Ramsay, 1978; Pande, 1975; Pande & Parvin, 1976). The properties of the outer face of the carrier have been investigated, but the measurement of K_m values for substrates within mitochondria is difficult. A K_m value of 5 mM (4 nmol/mg of protein) was determined for carnitine in the matrix of ox heart mitochondria after manipulating the internal carnitine concentration (Tubbs & Ramsay, 1979), a value close to that for carnitine on the outside of the inner membrane (5.3 mM; Ramsay & Tubbs, 1975). A more direct approach is to use inverted particles prepared from mitochondria by ultrasonic treatment (Schulz & Racker, 1979). Substrates can be presented directly to what is normally the interior surface of the membrane, and thereby the properties of the matrix face of the carnitine exchange carrier can be investigated.

In order to measure exchange it is necessary to separate the particles from the surrounding medium, which has until now required tedious ultracentrifugation. More recently, Schulz & Racker (1979) used a faster procedure based on centrifugal filtration through Sephadex G-50. Here we report an even simpler method. In the presence of low concentrations of heparin, submitochondrial particles aggregate and are easily sedimented in a micro-centrifuge. This effect of heparin makes the study of transport processes in submitochondrial particles much easier.

EXPERIMENTAL

Materials

L-[^{14}C]Carnitine was prepared from DL-[^{14}C]carnitine as described by Ramsay & Tubbs (1976). The carnitine transport inhibitor 11-trimethylaminoundecanoyl-DL-carnitine was synthesized from 11-trimethylamino-undecanoic acid and DL-carnitine (Ramsay & Tubbs,

1976), but a better method for the synthesis of the acid was used. 11-Bromoundecanoic acid (100 mmol; Aldrich Chemical Co.) was dissolved in 100 ml of methanol, mixed with 80 ml of aq. 25% (w/v) trimethylamine (320 mmol) and left at room temperature for 72 h. Methanol was removed by rotary evaporation, and the syrup was dissolved in water and neutralized with KOH. The solution was applied to a column (5.5 cm $\times$ 36 cm) of Zerolit 225 SRA-14 sulphonate resin (H^+ form; Hopkin and Williams), and the product was eluted with 2 M- NH_3 solution. The eluate was dried *in vacuo* and the 11-trimethylaminoundecanoic acid recrystallized from ethanol/diethyl ether.

Heparin (150 units/mg) was obtained from Evans Medical, Liverpool, U.K.

Submitochondrial particles

Ox heart mitochondria were prepared as described previously (Ramsay & Tubbs, 1975). Frozen mitochondria (600 mg of protein in 10 ml) were thawed and diluted with 10 ml of 0.3 M-sucrose containing 1 mM-EGTA and 10 mM-Mops adjusted to pH 7.2 with KOH (sucrose medium); MgCl_2 (15 mM) and 20 mM-L-carnitine (as the free zwitterion) were also added. The suspension was sonicated for 4 min (eight periods of 30 s) at an output of 400 W (Rapidis A600G sonicator; Ultrasonics Ltd., Otley Road, Shipley, Yorks., U.K.) in a metal bath cooled by ice/water. Unbroken mitochondria and debris were removed by centrifugation at 28000 g for 10 min, and the submitochondrial particles were harvested from the supernatant (100000 g for 45 min). The pellets were resuspended in sucrose medium and applied to a column (2.5 cm $\times$ 23 cm) of Sepharose CL-6B (Pharmacia) equilibrated with sucrose medium to separate the particles from the excess carnitine.

The carnitine-loaded particles were incubated with 3 mM-ATP, 3 mM- MgCl_2 and 200 μM -L-[^{14}C]carnitine (10 Ci/mol) at 15 $^\circ\text{C}$ for 1.5 h. Excess L-[^{14}C]carnitine was clearly separated from the particles by gel filtration

Fig. 1. Sedimentation of submitochondrial particles in the presence of heparin

Submitochondrial-particle suspension (20 µl; 0.3 mg of protein) loaded with L-[¹⁴C]carnitine was added to 330 µl of sucrose medium containing 3 mM-ATP, 3 mM-MgCl₂ and 0–1.0 mg of heparin/ml with (stippled columns) or without (blank columns) 10 mM-11-trimethylamino-undecanoyl-DL-carnitine. The samples were centrifuged for 1 min in a Microfuge and 300 µl of the supernatant was counted for radioactivity. The radioactivity in the supernatant with 5–50 µg of heparin/ml is due to extra-particulate [¹⁴C]carnitine.

through Sepharose CL-6B. The particles were again sedimented in the ultracentrifuge and resuspended in sucrose medium containing 3 mM-ATP and 3 mM-MgCl₂ to a volume of 2 ml. The suspension was assayed for carnitine, protein and radioactivity (Ramsay & Tubbs, 1975). The yield of protein as a percentage of the mitochondrial protein was usually about 10%. The carnitine content of the particles was normally about 4 nmol/mg of protein. The internal space, measured as described by Haddock *et al.* (1970), was usually 0.5 µl/mg. The particles were stored in liquid N₂. We have found that the particles loaded with L-[¹⁴C]carnitine may be stored in liquid N₂ for at least 1 month and that the freezing and thawing entailed causes only a slight leakage of radioactivity.

RESULTS AND DISCUSSION

It is generally accepted that vesicles obtained by sonication of mitochondria are mostly inverted, so that the matrix surface (M-side) of the inner membrane faces outwards (Racker, 1970; Chua & Shrago, 1977). To confirm the inversion of our particles, we measured the inhibition of adenine nucleotide transport (Chua & Shrago, 1977). The exchange of ADP in submitochondrial particles loaded with [¹⁴C]ADP was decreased by less than 10% by 20 µM-atractylate, but was virtually completely inhibited by 2 µM-bongkrekate, demonstrating that particles were more than 90% inverted.

Studies of transport in submitochondrial particles require separation of the particles from the incubation medium, which is normally accomplished by ultracentri-

fugation, an inconvenient method, or by filtration through an appropriate membrane, which may jeopardize the integrity of the particles. Rapid filtration through Sephadex G-50 has been used for uptake experiments (Schulz & Racker, 1979), but, for experiments where the exchange of radioactivity out of the particles is measured, sedimentation of the particles in the presence of heparin has proved even more convenient. The method depends on the ability of heparin to promote aggregation of the particles so that they sediment after 1 min centrifugation in a Minifuge. As in precipitation of lipoproteins by heparin, the aggregation is probably the result of the formation of insoluble complexes between the sulphated polysaccharide and the lipoprotein surface of the particles (Cornwell & Kruger, 1961). The effect is promoted by the presence of bivalent cations (here 3 mM-Mg²⁺) and by sucrose (Burstein *et al.*, 1970).

Fig. 1 shows the dependence of the precipitation of the particles on the heparin concentration. In the absence of heparin, no pellet was formed, so that all the radioactivity remained in the supernatant. We have routinely used a heparin concentration of 10 µg/ml, well below the concentration that causes a leak of carnitine from the particles (Fig. 1). The radioactivity appearing in the supernatant at high concentrations of heparin may be partly due to a leak of carnitine mediated by the carnitine carrier, because it is partly inhibited by 11-trimethylaminoundecanoyl-DL-carnitine, a competitive inhibitor of carnitine exchange (Ramsay & Tubbs, 1976; Tubbs *et al.*, 1980). It should be noted that mersalyl (Pande &

Fig. 2. Carnitine exchange in L-[¹⁴C]carnitine-loaded submitochondrial particles and its inhibition by 11-trimethylaminoundecanoyl-DL-carnitine

Sucrose medium (0.3 ml) containing 3 mM-ATP, 3 mM-MgCl₂ and 10 mM-L-carnitine was stirred at 25 °C in a Minifuge tube. Exchange was initiated by the addition of 20 µl of submitochondrial-particle suspension (0.32 mg of protein, containing 5.2 nmol of L-carnitine/mg) and stopped by addition of 30 µl of 11-trimethylaminoundecanoyl-DL-carnitine plus heparin to give final concentrations of 20 mM and 10 µg/ml respectively. The suspension was centrifuged for 1 min in a Quickfit micro-centrifuge (15000 g), and 300 µl of the supernatant was counted for radioactivity. Additions were 10 mM-L-carnitine (○), 10 mM-11-trimethylaminoundecanoyl-DL-carnitine (■), both of these (●) or none (i.e. leak) (□).

Fig. 3. Double-reciprocal plot for release of L-[^{14}C]carnitine from submitochondrial particles induced by L-carnitine or palmitoyl-L-carnitine

Experimental conditions are described in Fig. 2. Additions were 3–20 mM-L-carnitine (○) or 25–150 μM -palmitoyl-L-carnitine, with bovine serum albumin in the proportion of 40 $\mu\text{g}/\mu\text{mol}$ of palmitoyl-L-carnitine (●).

Parvin, 1976) was not a suitable stop inhibitor in this system because it prevented the heparin-induced aggregation of the particles.

The technique was used to study the efflux of L-[^{14}C]carnitine from submitochondrial particles. Radioactivity was released by exchange with external L-carnitine (Fig. 2) or with palmitoyl-L-carnitine, but its leak was very slow in the absence of external substrate. The exchange was inhibited by 11-trimethylaminoundecanoic acid, but higher concentrations were required than those used to stop exchange in mitochondria. Efflux was measured in the presence of various concentrations of L-carnitine and of palmitoyl-L-carnitine (Fig. 3). From the double-reciprocal plots (Fig. 3), the apparent K_m values for L-carnitine (8 mM) and for palmitoyl-L-carnitine (150 μM) were the same as those observed in mitochondria. Although the maximum rate of exchange is the same for both substrates, the specific activity was usually less than 10% of the rate observed in mitochondria, implying that

much of the exchange activity is lost during sonication. Different particle preparations have shown very dissimilar rates of carnitine exchange or leak; however, these appear to vary together, supporting the suggestion (Ramsay & Tubbs, 1976) that the same carrier mediates both.

The methods described in the present paper provide a convenient system for studying the matrix face of the carnitine-exchange system or of any other mitochondrial transport process. The experiments strongly suggest that the external and matrix faces of the carnitine-exchange carrier, when studied in isolation, have very similar properties. However, *in vivo*, it is likely that the different properties of the outer and inner carnitine palmitoyl-transferases and the existence of two matrix pools of carnitine (Murthy & Pande, 1984) serve to bias the exchange in favour of the transport of fatty acids into the mitochondria.

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