TRANSLOCATION OF LONG CHAIN FATTY ACIDS INTO LECITHIN LIPOSOMES CONTAINING THE LONG CHAIN FATTY ACYL-CoA SYNTHETASE

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1. Introduction

Transport of long chain fatty acids through biological membranes was reported to be mediated either by adsorption to the cell membrane followed by simple diffusion [1,2], carrier-facilitated transport [3,4] or translocation catalyzed by long chain fatty acyl-CoA synthetase [5–7]. The simple diffusion of long chain fatty acids requires flip–flop of the transported acid within the bilayer membrane. However, the flip–flop diffusion rate for free fatty acids was found to be relatively low [8,9] to account for the high metabolic turnover of long chain fatty acid used as substrate in vivo [10], in organ perfusion [11] or in cultured cells [12]. The possible role of a specific protein carrier in facilitated diffusion of long chain fatty acids is still unresolved due to methodological difficulties presented by the nonspecific absorption of long chain fatty acids to biological membranes, the limited concentration range of free fatty acid which may be used for binding studies in the absence of added detergent, as well as the micellar character of the fatty acid substrate in the presence of added detergent. Vectorial translocation of long chain fatty acids catalyzed by the ATP-dependent long chain fatty acyl-CoA synthetase (EC 6.2.1.3) was first suggested [5] in *Escherichia coli* K-12. This claim was based on the inability of a mutant lacking the acyl-CoA synthetase to take up fatty acids, the absence of an efflux reaction for the entrapped fatty acid moiety and the substrate specificity for uptake as compared to the substrate specificity for the synthetase reaction. However, it is worth noting that the isolated permeation step was not analyzed specifically but was followed in terms of total uptake as reflected in esterification or oxidation. Whatever the mode of carrier-mediated transport, one still expects the overall metabolic pathway to be enzyme-dependent, subjected to saturation kinetics, as well as being substrate specific. Hence, the claim for vectorial translocation by acylation of long chain fatty acids requires the analysis of the transport process under conditions avoiding further metabolism. Liposomes containing the long chain fatty acyl-CoA synthetase have been used here for analyzing the role of the synthetase in vectorial translocation of long chain fatty acid. The implication of this study for long chain fatty acid translocation through various biological membranes is discussed.

2. Methods

2.1. *Preparation of liposomes*

Lecithin (20 mg) in chloroform was evaporated to dryness under a stream of N_2 and kept under vacuum for 12 h. The dried lecithin was suspended in 2 ml medium composed of 1% glycerol, 1 mM DTT, 1 mM $MgSO_4$, 0.5 mM EDTA, 5 mM Tris–HCl (pH 6.8) and 120 mM K-phosphate (pH 6.8); 1 mg purified rat liver long-chain fatty acyl-CoA synthetase (EC 6.2.1.3) was added and the suspension was passed 4 times through a French press (20 000 lb/in.^{2}, 1 in. diameter piston) [13]. Of the resulting suspension 1.0 ml was applied onto a 1.0 × 30 cm Sepharose 4-B column, equilibrated with 10 mM Tris–HCl buffer (pH 7.4), 1 mM DTT, 0.02% azide and 100 mM NaCl. The column was eluted with the same buffer and fractions of 1 ml were collected and analyzed for A_{300} as well as for long-chain fatty acyl-CoA synthetase activity [14]. The activity peak eluted with the void volume correlates with the A_{300} and contains all of the lecithin

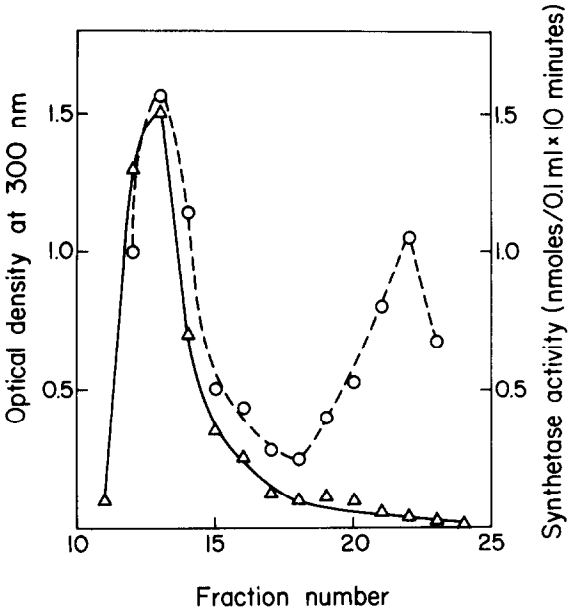

Fig.1. Preparation of synthetase-liposomes: A Sepharose-4B profile. Lecithin liposomes containing the long-chain fatty acyl-CoA synthetase were prepared as in section 2. (△) A_{300}; (○) long chain fatty acyl-CoA synthetase activity (nmol palmitoyl-CoA formed/0.1 ml fraction × 10 min).

loaded onto the column, thus representing liposome-entrapped long chain fatty acyl-Coa synthetase (fig.1).

2.2. *Determination of liposomal internal volume*

Liposomes were prepared as above in the presence of 17 mM added ATP. Subsequent to the elution of the liposomal fraction from Sepharose 4-B, the ATP content was determined by following the bioluminescense in the presence of luciferin–luciferase [15], using added ATP as an internal standard. No ATP could be detected in the liposomal fraction, unless Triton was added to 0.5% final conc. The ATP measured in the presence of added Triton was thus considered to be internal ATP and the respective dilution factor served for the calculation of the internal liposomal volume. The calculated internal volume was referred to the lecithin content of the liposomal fraction. Liposomes containing synthetase prepared as described here were found to have an internal volume of 0.5 μl/μmol lecithin. These liposomes were found to be non-leaky to ATP at 0°C for 24 h, or at 30°C for 20 min. However, upon subjecting the liposome fraction to repeated freezing and thawing, 80% of the ATP content could be detected even in the absence of added Triton with a concomitant decrease in the internal volume of the liposome fraction.

Purified rat liver long chain fatty acyl-CoA synthetase was prepared and assayed as in [14]. The last gel filtration step was omitted. The specific activity of the purified preparation amounted to 50–100 nmol/mg × min. Palmitoyl-CoA hydrolase was purified from *E. coli* K-12, grown in minimal medium supplemented with 0.4% glucose and 10 mM Na-acetate as in [16]. The streptomycin step was omitted and the second ammonium sulfate fraction was used throughout. Palmitoyl-CoA hydrolase activity was measured either colorimetrically (during preparation) as in [16] or by heptane partitioning as in [14]. The specific activity amounted to 1 μmol/mg × min. The purified fraction did not hydrolyze lecithin into either phosphatidic acid, diglyceride or free fatty acid. [^{3}H]-palmitoyl-CoA was prepared enzymatically as in [17]. Lecithin (Makor Chemicals) was assayed by its organic phosphate content [18].

3. Results

Liposomes containing the long chain fatty acyl-CoA synthetase purified from rat liver microsomes were found to catalyze fatty acyl-CoA formation in the presence of added ATP, CoA and palmitic acid. Of the fatty acyl-CoA formed by the synthetase containing liposomes 30–50% could be co-eluted with the liposomal fraction upon subjecting the reaction mixture to Sepharose 4-B gel filtration (fig.2, fractions 9–16), whereas 50–70% of the acyl-CoA formed under these conditions was co-eluted with the albumin fraction (fig.2, fractions 18–24). The accessibility of the eluted acyl-CoA product to hydrolysis by palmitoyl-CoA hydrolase served as a measure for the acyl-CoA fraction entrapped within the synthetase-liposomes. Thus, 60% of the acyl-CoA coeluted with the liposomal fraction escaped hydrolysis in the absence of added Triton, whereas virtually all of the acyl-CoA co-eluted with albumin was found to be hydrolyzed under the same conditions. On the other hand, preformed acyl-CoA preincubated with the synthetase-liposomes was found to be almost completely accessible to acyl-CoA hydrolase even in the absence of added Triton (table 1). Hence, the acyl-CoA fraction entrapped within the synthetase-liposomes reflects the transport of the acyl-CoA product mediated by the liposomal long-chain fatty acyl-CoA synthetase in the course of catalysis.

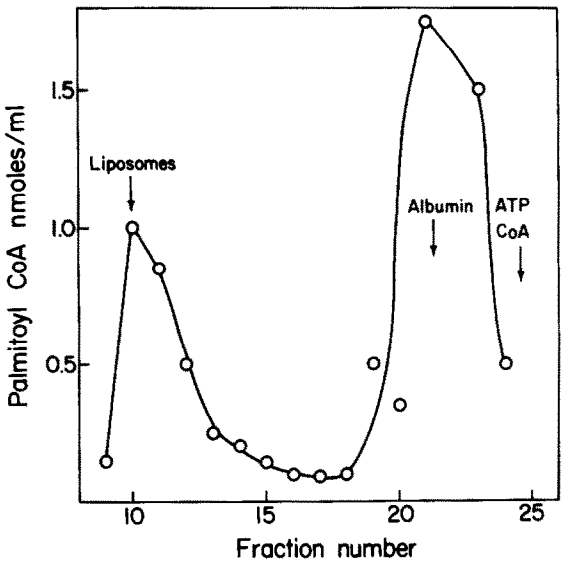

Fig.2. Palmitoyl-CoA formed by synthetase-liposomes: A Sepharose-4B profile. Synthetase liposomes prepared as in section 2 were incubated for 10 min at 30°C in the presence of 50 mM NaCl, 0.01% azide, 150 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1 mM DTT, 50 mM $MgCl_2$, 0.05% Triton X-100, 0.4 mM CoA, 20 mM ATP, 3.2 mg albumin/ml and 0.2 mM palmitate. An aliquot of the reaction mixture was directly assayed for palmitoyl-CoA content and the rest was applied onto a 2.5 × 30 cm Sepharose-4B column. The column was eluted with column buffer (see section 2) and palmitoyl-CoA was determined in 1 ml fractions. Total palmitoyl-CoA applied to the column amounted to 14 nmol.

The concentration of the entrapped acyl-CoA fraction could be calculated from the liposomal internal volume and the amount of acyl-CoA resistant to hydrolysis in the presence of added acyl-CoA hydrolase. Thus, the internal concentration of acyl-CoA amounted to 3 μmol/ml, as compared to 4 nmol/ml for the exogenous acyl-CoA concentration. Hence, translocation of long-chain fatty acids catalyzed by the synthetase liposomes builds up an apparent acyl-CoA gradient of 750 under incubation conditions as described under fig.2.

The polarity of acyl-CoA formation as catalyzed by synthetase liposomes was studied by following the translocated acyl-CoA fraction under conditions whereby the various substrates of the synthetase reaction were allowed to react on the two opposing faces of the bilayer membrane. This was accomplished by preparing synthetase liposomes containing internally entrapped substrates at concentrations of ATP and CoA high enough to ensure the detection of palmitoyl-CoA if formed. As shown in table 2, out of all the permutations studied, the only combination which yielded palmitoyl-CoA was observed under conditions whereby all 3 reactants were present at the same face of the bilayer membrane.

Table 1
Palmitoyl-CoA accessibility to hydrolysis by palmitoyl-CoA hydrolase (nmol)

Source of palmitoyl-CoA	Total	Hydrolyzed in the absence of added Triton	Hydrolyzed in the presence of 0.5% Triton
Formed by synthetase-liposomes, coeluted with liposomes (fig.2, fraction 10)	1.42	0.57	1.41
Formed by synthetase-liposomes, coeluted with albumin (fig.2, fraction 22)	1.70	1.45	1.58
Pre-formed	2.0	1.74	1.68

Palmitoyl-CoA fractions formed by synthetase-liposomes and fractionated on Sepharose 4-B were prepared as in fig.2. Aliquots (0.5 ml) of fractions 10 and 22 (fig.2) were incubated for 10 min at 30°C with 18 μl palmitoyl-CoA hydrolase in the absence and presence of added Triton X-100. Palmitoyl-CoA hydrolysis was determined as in section 2. For analyzing the optional translocation of preformed palmitoyl-CoA into synthetase-liposomes, 2 μmol palmitoyl-CoA were incubated for 10 min at 30°C with 1.5 ml synthetase-liposomes and subjected then to hydrolysis by added palmitoyl-CoA hydrolase as above

Table 2
Polarity of synthetase-liposomes

Internally entrapped substrates	Externally added substrates	Palmitoyl-CoA formed (nmol)
CoA, 5 mM	ATP, 18 mM + CoA, 0.4 mM	60.0
CoA, 5 mM	ATP, 18 mM	0.5
ATP, 18 mM	CoA, 0.4 mM	0.0
CoA, 5 mM + ATP, 18 mM		0.0

Synthetase liposomes were prepared in a medium composed of 120 mM K-phosphate (pH 6.8), 5 mM Tris–HCl (pH 7.4), 0.5 mM EDTA, 1 mM DTT, 1% glycerol, 30 mM $MgCl_2$, 0.05% Triton X-100 and 1.6 mg albumin/ml, with the addition of either CoA, ATP, or both, as stated. The 3 species of liposomes thus formed were filtered through Sepharose 4-B column and the respective synthetase-liposome fractions were incubated for 20 min at 30° C in the presence of 50 mM NaCl, 0.01% azide, 150 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1 mM DTT, 50 mM $MgCl_2$, 0.05% Triton X-100, 3.2 mg albumin/ml and 0.2 mM palmitate, supplemented with either ATP, CoA, or both, as stated. Palmitoyl-CoA formed was determined as in section 2

4. Discussion

Vectorial translocation of long chain fatty acids into lecithin liposomes was found here to be catalyzed by rat liver microsomal long-chain fatty acyl-CoA synthetase. Entrapment of the acyl-CoA is not due to transport of preformed acyl-CoA produced at the outer face of the synthetase liposomes. Neither is it the result of a simple palmitate diffusion followed by intraliposomal synthetase catalysis. The requirement for all 3 substrates of the synthetase reaction to be present at the outer face of the liposomal membrane, taken together with the observed impermeability to nucleotides exclude this possibility. Hence, transport of long-chain fatty acids in the synthetase liposomes reflects vectorial translocation of the fatty acid substrate mediated by the liposomal fatty acyl-CoA synthetase in the course of catalysis. It is worth noting that the model system analyzed here follows the translocation step specifically, thus avoiding deductions concerning the mode of transport of long-chain fatty acid based on analysis of the whole metabolic pathway for fatty acid.

Translocation through the plasma membrane is under the constraint of having the ATP and CoA nucleotides on the interior face of the bilayer with the fatty acid substrate present on the other face of the same membrane. Hence, the requirement for all 3 substrates of the synthetase reaction to be present on the same face of the liposomal bilayer membrane seems to contraindicate translocation through the plasma membrane as the mode of transport of long chain fatty acid into the interior of the cell. However, it should be pointed out that the specific polarity found here may stem from the specific lipid microenvironment prevailing within the lecithin liposome, being at variance with the respective synthetase lipid environment of the native plasma membrane. Alternatively, the polarity of the plasma membrane synthetase may prove to be different from that of the microsomal synthetase. Hence, the polarity of synthetase-liposomes as a function of the lipid species as well as of the specific synthetase used has still to be elucidated before drawing conclusions concerning the relevance of translocation for the transport of long-chain fatty acid through the plasma membrane.

The polarity as observed here may bear relevance to the transport of long chain fatty acid from the cytosol into cellular subparticles, i.e., mitochondria. Carnitine acyltransferase has been claimed to be rate-limiting for long-chain fatty acid oxidation in isolated mitochondria [19]. However, as shown in [20], the uptake of palmitate (6.5 μM) into rat liver mitochondria in the presence of added ATP and CoA and in the absence of carnitine amounted to 50% of that observed in the presence of added carnitine. At higher palmitate concentration (16.3 μM), the obligatory requirement for added carnitine was even less obvious. These results confirm the observations in [21] concerning the long-chain fatty acyl-CoA synthetases of the outer and inner mitochondrial membranes. Comparing the kinetics of the two enzymes with that of long chain fatty acid oxidation has indicated that under conditions of fatty acid oxidation at low palmitate concentration, the outer synthetase is operating (low K_m for palmitate) and carnitine is obligatory for transport of the acyl-CoA product through the inner membrane. However, at high concentration of long-chain fatty acid, the activation reaction is catalyzed by the inner membrane synthetase (high K_m for palmitate) leading to comparable oxidation rates for palmitate in the absence of added carnitine [21]. Hence, carnitine acyltransferase seems to be obligatory for mediating the translocation of long-chain fatty acyl-CoA once formed outside the inner impermeable membrane, but not

for the transport of long-chain fatty acid under conditions suitable for its activation by the inner membrane synthetase (ATP, CoA, high palmitate). It is suggested that palmitate transport in the absence of carnitine may be mediated by translocation catalyzed by the inner synthetase having a polarity similar to that observed for the lecithin liposomes studied here.

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