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Nonspecific lipid transfer protein (sterol carrier protein 2) is bound to rat liver mitochondria: its role in spontaneous intermembrane phospholipid transfer

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In the present study we have investigated the transfer of phospholipids between vesicles and rat liver mitochondria. Transfer was measured by electron paramagnetic resonance spectroscopy using vesicles that contained spin-labeled phospholipids. A spontaneous transfer was observed which could be strongly inhibited by treating the mitochondria with the thiol reagent mersalyl. Transfer was also greatly reduced after a saline wash of the mitochondria; the transfer activity was then recovered in the wash. This activity was inhibited by tryptic digestion and mersalyl. By gel chromatography, enzyme immunoassay and immunoblotting it was demonstrated that the activity in the wash was due to the nonspecific lipid transfer protein (sterol carrier protein 2). We could estimate that up to 85% of the spontaneous phospholipid transfer between vesicles and rat liver mitochondria was mediated by this transfer protein.

Introduction

Since its first observation in simple phospholipid vesicle systems [1,2] the spontaneous phospholipid transfer between membranes has received wide attention. It has been firmly established that this process occurs via a soluble monomer diffusion system [3–5], driven by the desorption of a lipid molecule from the vesicles [4] and governed by the hydration free energy of the monomers [5].

Phospholipid transfer in the absence of added transfer proteins has also been observed in systems involving natural membranes, including erythrocytes [5], whole bacterial cells [6,7] and subcellular organelles such as microsomes and mitochondria [8–11]. In contrast to model membranes and intact cell systems, the spontaneous transfer between subcellular membranes was observed to proceed rapidly. In previous studies we have investigated this process by an EPR assay measuring the transfer between vesicles containing spin-labeled phospholipids and rat liver mitochondria [12,13]. We observed that the transfer of spin-labeled phospholipids was faster with mitochondria than with vesicles as acceptor membranes [12], that the transfer increased with the concentration of mitochondrial membranes [13] and that the transfer was inhibited by *N*-ethylmaleimide [12]. The latter observation suggested

Abbreviations: EPR, electron paramagnetic resonance; spin-labeled PC, 1-acyl-2-(12-doxylstearoyl)-*sn*-glycero-3-phosphocholine; spin-labeled PE, 1-acyl-2-(12-doxylstearoyl)-*sn*-glycero-3-phosphoethanolamine; mersalyl, *O*-[(3-(hydroxymercuri)-2-methoxypropyl)carbamoyl]phenoxyacetic acid.

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that the transfer was dependent on a mitochondrial membrane protein containing an essential thiol group.

Here we have further investigated the transfer activity present in the mitochondria. It will be demonstrated that this activity is mainly due to the nonspecific lipid transfer protein (i.e. sterol carrier protein 2) bound to the mitochondria.

Materials and Methods

Materials. PC, PE and lyso-PE (egg) were purchased from Sigma; 12-doxylstearic acid was supplied by Aldrich; spin-labeled PC was synthesized according to Boss et al. [14], and spin-labeled PE was prepared from spin-labeled PC by base exchange with phospholipase D (EC 3.1.4.4) from cabbage (Boehringer) [15]. Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were also obtained from Boehringer. Ultrogel AcA-54 for gel chromatography was purchased from LKB Bromma. Mersalyl was from Sigma and HRP Color Development Reagent from Bio-Rad.

Preparation of phospholipid vesicles. Mixtures of spin-labeled and natural phospholipids (5–15 mM phospholipid phosphorus) were suspended in 20 mM Tris-HCl/5 mM EDTA (pH 7.4) and sonicated with a Branson sonifier (50 W output) for 15–20 min at intervals in an ice bath under N_2. The resulting suspension was chromatographed on Sepharose 4B according to Huang [16]. The second half of the second peak was collected [17] and the pooled fractions were concentrated on an Amicon ultrafilter (equipped with an XM-100 membrane) to a concentration ranging between 5 and 15 mM phospholipid. Phospholipid content was assayed by the method of Nakamura [18].

Preparation of mitochondria. A 30% (w/v) homogenate was prepared from rat liver in 0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl, pH 7.4 (buffer 1) using a Potter-Elvehjem tissue grinder. Cell debris and nuclei were sedimented at 3000 rpm for 10 min. Mitochondria were collected from the supernatant by sedimentation for 10 min at 7500 rpm and washed twice with buffer 1 (10 min at 12500 rpm). Salt-treated mitochondria were prepared by replacing the first wash with one of the following buffers: (a) 125 mM Tris-HCl/1 mM EDTA (pH 6.4, 7.4 or 8.4); (b) 125 mM KCl/10 mM Tris-HCl/1 mM EDTA (pH 6.4, 7.4 or 8.4); (c) 42 mM Na_2SO_4/125 mM sucrose/10 mM Tris-HCl/1 mM EDTA (pH 8.4). The mitochondria were used within 3 h after preparation. The protein content was determined by the biuret method.

Preparation of a mitochondrial extract. The crude mitochondrial preparation was washed with buffer 1 followed by washing with the KCl/Tris/EDTA buffer, pH 8.4 (12.5 ml per mitochondrial pellet from one liver). The clear yellowish supernatant was concentrated 5-fold by use of solid Aquacide (Calbiochem). Aggregated protein was removed by centrifugation (40000 rpm for 45 min) and the clear supernatant was dialyzed against 10 mM Tris-HCl/10% glycerol, pH 7.4. This preparation, containing about 4 mg protein/ml, will be referred to as mitochondrial extract. Protein was determined by the method of Lowry et al. [19].

Assay of phospholipid transfer activity. Phospholipid transfer activity was measured by the EPR method [12,20,21]. The incubation mixture contained donor vesicles (0.2 μmol phospholipid) consisting of spin-labeled PC/PC (1 : 1, molar ratio) and acceptor mitochondria (4 mg protein) in a volume made up to 0.3 ml with buffer 1. To determine the transfer activity in the mitochondrial extract, spin-labeled donor vesicles (0.2 μmol phospholipid) of various compositions (see legends) were mixed with egg PC acceptor vesicles (0.9 μmol phospholipid) in a final volume of 0.3 ml. Incubations were carried out at room temperature.

Rates of spin-labeled phospholipid transfer were estimated from the increase of the low-field band second derivative with time. From calibration experiments this increase could be converted into nmoles of spin-labeled phospholipid transferred. Measurements were performed with the Varian E-109 EPR apparatus equipped with a data-acquisition system. Instrumental settings were: central field value, 3357 G; scan time, 2 h; time constant, 0.3 s. For second derivative operation, both high- and low-frequency modules were used, with the following settings, respectively: modulation amplitude, 5 and 10 G; modulation frequency, 100 and 1 kHz; receiver gain, 100 and 250. The klystron was tuned at a microwave frequency of approx. 9.44 GHz, with a microwave power of 20 mW.

Enzyme immunoassay. The content of nonspecific lipid transfer protein in the column eluates was estimated with a specific enzyme immunoassay [22].

Immunoblotting. Identification of nonspecific lipid transfer protein in the mitochondrial extract was performed by immunoblotting as previously described [23]. Briefly, after incubation with specific anti-nonspecific lipid protein IgG the blot was transferred to a solution containing goat anti-rabbit IgG conjugated to horseradish peroxidase (1 : 2000 dilution). Peroxidase activity was visualized with 0.5 mg/ml HRP Color Development Reagent (Bio-Rad), 16.7% (v/v) methanol and 0.015% (v/v) H_2O_2. Reaction was stopped by washing in distilled water.

Results

Effect of mersalyl treatment

In a previous study [12] a rapid transfer of spin-labeled phospholipid was observed between vesicles and rat liver mitochondria. This transfer was inhibited when the mitochondria were treated with *N*-ethylmaleimide. The effect of another thiol-blocking reagent, i.e. mersalyl [24], on this transfer is shown in Fig. 1. Treatment of the mitochondria with 0.65 mM mersalyl (10 min, room temperature) gave a maximum inhibition of 65%. Incubation of the mersalyl-treated mitochondria with 6.5 mM cysteine or dithioerythritol (10 min, room temperature) restored the transfer activity to 84% and 91%, respectively. These data strongly suggest that a mitochondrial SH-group is involved in the transfer reaction.

Effect of salt treatment

In order to investigate the interaction of the transfer activity with the mitochondria, the mitochondria were washed with salt-containing buffers of different pH (see Materials and Methods). As shown in Table I, treatment with Tris-HCl (125 mM), KCl (125 mM) and Na_2SO_4 (42 mM) strongly reduced the phospholipid transfer to these mitochondria. This reduction, varying between 65% and 85%, appears to depend on the ionic strength of the buffers used. A slight dependency upon pH is also apparent, the reduction of transfer being more pronounced at alkaline pH (e.g.

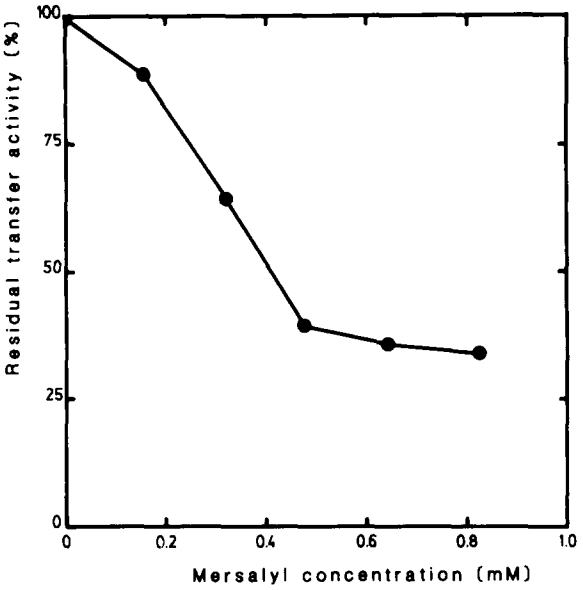

Fig. 1. Phospholipid transfer between donor vesicles and mitochondria treated with mersalyl. Mitochondria (4 mg protein) were preincubated at room temperature for 10 min with the indicated concentrations of mersalyl. Upon addition of donor vesicles consisting of 200 nmol spin-labeled PC/PC (1 : 1 molar ratio) transfer of spin-labeled PC was measured as described in Materials and Methods.

87% against 75% for KCl at pH 8.4 and 6.4, respectively). After the salt treatment the mitochondria to be tested in the transfer assay were washed with buffer 1 to eliminate the effect of any salt remaining in the mitochondrial preparation. This implies that the reduced transfer observed was probably due to the removal of protein(s) bound to the mitochondria.

Phospholipid-transfer activity in the mitochondrial extract

The mitochondrial extract obtained by treatment of mitochondria with KCl-Tris-EDTA buffer (see Materials and Methods) was assayed for phospholipid transfer activity. Transfer of spin-labeled PC to KCl-treated mitochondria was measured. As shown in Fig. 2, spontaneous transfer to these mitochondria was very low (i.e. initial rate of uptake 0.7 nmol spin-labeled PC/min; curve A). However, upon addition of the mitochondrial extract (0.6 mg protein) transfer was greatly enhanced (i.e. 4.7 nmol spin-labeled PC/min; curve B) and comparable to the transfer

TABLE I

EFFECT OF SALT-TREATMENT ON TRANSFER OF SPIN-LABELED PC FROM VESICLES TO MITOCHONDRIA

Mitochondria were washed with different buffers as described in Materials and Methods and used as acceptor membranes in the EPR transfer assay. The resutls are expressed as percentages of the initial spin-labeled PC transfer rate to normal acceptor mitochondria.

Salt treatment of mitochondria at pH:	6.4	7.4	8.4	No. of Expts.
None	100	100	100	2
125 mM Tris-HCl/1 mM EDTA	33	14	13	3
125 mM KCl/10 mM Tris-HCl/1 mM EDTA	25	18	13	3
42 mM Na_2SO_2/125 mM sucrose/10 mM Tris-HCl/1 mM EDTA	–	–	22	1

observed with untreated mitochondria as acceptor membranes (i.e. 5.4 nmol spin-labeled PC/min; curve C).

The phospholipid transfer activity in the mitochondrial extract was also assayed with egg PC vesicles as acceptor membranes. The donor vesicles consisted of spin-labeled PC/PC/PE (1 : 1 : 1, molar ratio) and spin-labeled PE/PC (1 : 2, molar ratio). In the absence of mitochondrial extract the transfer of spin-labeled PC and spin-labeled PE was 0.6 and 0.2 nmol/min, respectively, (curves A, Fig. 3) and comparable to the rate observed with KCl-treated mitochondria (curve A, Fig. 2). Addition of mitochondrial extract (0.6 mg protein) accelerated the transfer of both spin-labeled PC and spin-labeled PE, yielding initial rates of 6.6 and 5.3 nmol/min, respectively. Treatment of the mitochondrial extract with mersalyl (5.7 mM, 10 min at room temperature) inhibited the transfer of spin-labeled PC and spin-labeled PE by 52% and 64%, respectively (Table II). Transfer activity was almost completely restored by further incubation of the mitochondrial extract with cysteine (57 mM). These experiments demonstrate that the transfer activities bound to the mitochondria and released in the extract have the same sensitivity towards thiol-blocking reagents. Transfer activity in the mitochondrial extract was inhibited by trypsin and chymotrypsin, confirming the protein nature of the activity (Table II).

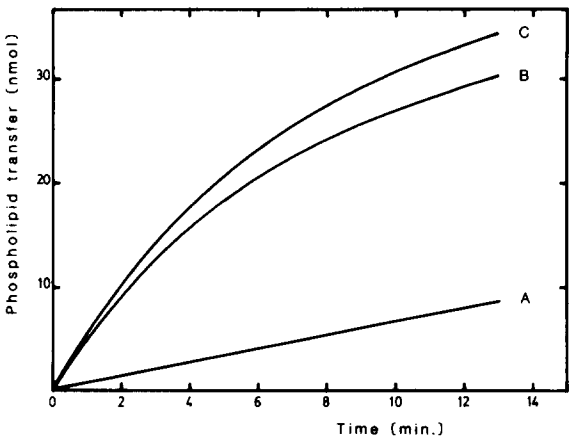

Fig. 2. Effect of mitochondrial extract on the transfer of spin-labeled PC from donor vesicles to KCl-treated mitochondria. A, KCl-treated mitochondria; B, KCl-treated mitochondria with 150 μl (0.6 mg protein) of mitochondrial extract; C, untreated mitochondria. For further details see Materials and Methods.

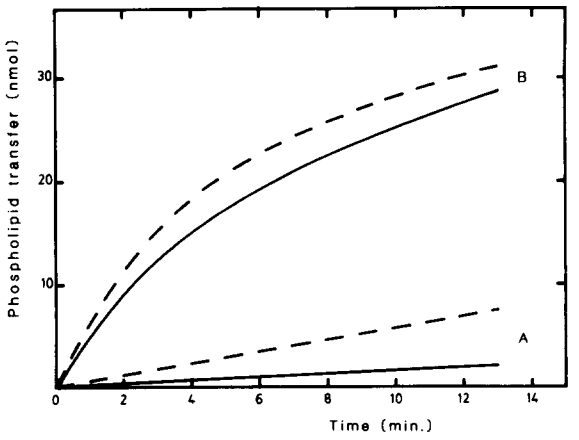

Fig. 3. Transfer of spin-labeled phospholipids between donor and acceptor vesicles. The incubation mixture consisted of 200 nmol donor vesicles comprised of spin-labeled PE/PC (1 : 2, molar ratio; solid lines) or spin-labeled PC/PC/PE (1 : 1 : 1, molar ratio; dashed lines) and 900 nmol egg PC acceptor vesicles, in the absence (A) or presence (B) of 150 μl (0.6 mg protein) mitochondrial extract.

TABLE II

EFFECT OF MERSALYL AND PROTEOLYTIC ENZYMES ON THE PHOSPHOLIPID TRANSFER ACTIVITY OF THE MITOCHONDRIAL EXTRACT

The vesicle-vesicle EPR assay was performed with donor vesicles consisting of spin-labeled PC (1:1, molar ratio) or spin-labeled PE/PC (1:2, molar ratio) in the presence of 150 μl mitochondrial extract (0.6 mg protein) subjected to the following treatments: [a] 5.7 mM mersalyl for 10 min at 37°C; [b] as in a followed by 10 min incubation with 57 mM cysteine at 37°C; [c] trypsin (1 mg) for 120 min at 37°C; [d] chymotrypsin (1 mg) for 60 min at 37°C. The results are expressed as percentages of the initial spin-labeled PC or spin-labeled PE transfer rate to egg-PC acceptor vesicles in the presence of untreated mitochondrial extract.

Treatment	Relative rates of transfer		No. of Expts.
	spin-labeled PC	spin-labeled PE	
None	100	100	3
Mersalyl [a]	48	36	2
Mersalyl + cysteine [b]	88	95	2
None	100	100	3
Trypsin [c]	43	44	3
Chymotrypsin [d]	24	13	2

Identification of the mitochondrial phospholipid-transfer activity

There is ample evidence to conclude that the nonspecific lipid transfer protein is responsible for the PE-transfer activity present in the 105000 × *g* supernatant from rat liver [25,26]. The fact that the mitochondrial extract displayed PE-transfer activity suggested that nonspecific lipid transfer protein could be present. In order to investigate its occurrence the mitochondrial extract was fractionated on an AcA-54 column. The eluent was assayed for nonspecific lipid transfer protein with the enzyme immunoassay (Fig. 4, upper panel) and for PE-transfer activity (Fig. 4, lower panel). The bulk of the PE-transfer activity eluted in those fractions (Nos. 52–62) that contained specific lipid transfer protein. In this analysis the fractions were not heated, to ensure that PE-transfer activity possibly catalysed by a protein other than nonspecific lipid transfer protein could be identified. A small amount of this transfer activity was detected in the void volume. As immunoreactive material was also found in these fractions, we believe that this transfer activity results from aggregated nonspecific lipid transfer protein. To provide additional evidence, immunoblotting of the mitochondrial extract was performed using the affinity-purified antibody against rat liver nonspecific lipid transfer protein. As shown in Fig. 5, the blot gave one band of apparent molecular weight 14000, indicating the presence of nonspecific lipid transfer protein in the mitochondrial extract. The eluent of the AcA-54 column was also assayed for PC-transfer activity. It was shown that this activity coeluted with PE-transfer activity (data not shown). This strongly suggests that apart from nonspecific lipid transfer protein no other

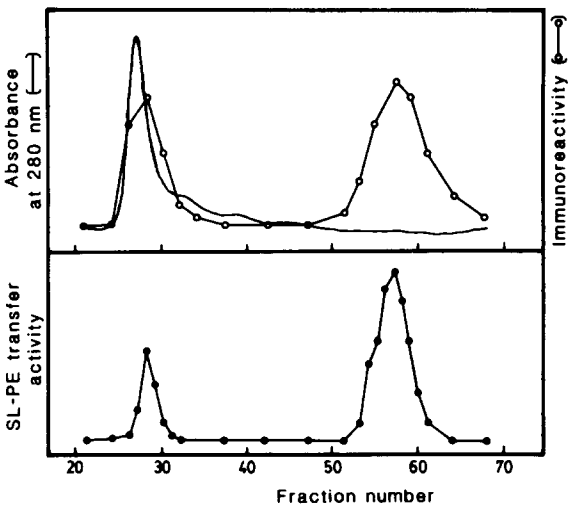

Fig. 4. Molecular sieve chromatography of the mitochondrial extract on an AcA-54 column. Upper panel presents the elution profile of the protein and the distribution of immunoreactive material; lower panel, distribution of spin-labeled PE (SL-PE) transfer activity. For further experimental details, see Materials and Methods.

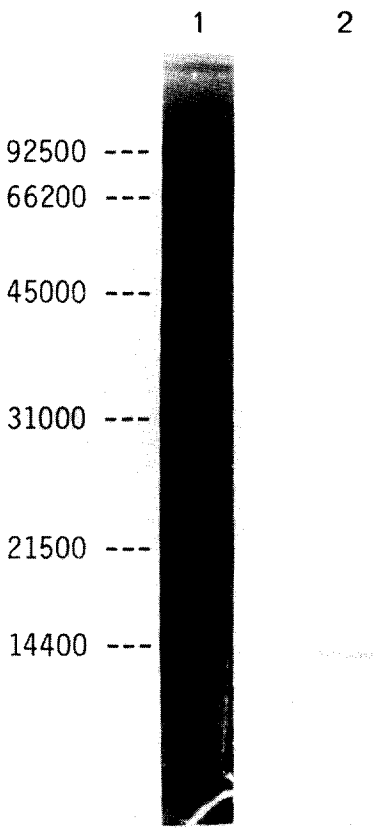

Fig. 5. Detection of nonspecific lipid transfer protein in the mitochondrial extract by immunoblotting. For experimental details, see Materials and Methods. Lane 1, marker proteins of molecular weights as indicated; lane 2, immunoblot of 350 μg of mitochondrial extract protein (see Materials and Methods).

lipid transfer proteins are present in the mitochondrial extract.

Discussion

In the present study it is demonstrated that the 'spontaneous' transfer of spin-labeled PC and PE between rat liver mitochondria and vesicles is mainly due to nonspecific lipid transfer protein adhering to these mitochondria. In a previous study we have estimated by enzyme immunoassay that mitochondrial preparations contain 0.050 ± 0.005 μg nonspecific lipid transfer protein per mg mitochondrial protein [22]. Here we show that this protein is tightly bound, as it cannot be removed by washing the mitochondria with buffer 1. However, the protein is released by washing with salt-containing buffer, indicating that the interaction between nonspecific lipid transfer protein and mitochondria is electrostatic by nature (Table I). In agreement with its basic character, isoelectric point of 8.6 [27], release was more efficient at pH 8.4 than at pH 6.4. The residual phospholipid transfer to the salt-treated mitochondria proceeds at approximately the same rate as observed with phospholipid vesicles as acceptor membranes (see Figs. 2 and 3 curves A). This strongly suggests that the residual transfer reflects a monomer diffusion mechanism, similar to that observed with vesicles [3–5].

In a series of studies by Stuhne-Sekalec and Stanacev [8–11] rapid 'spontaneous' transfers of both spin-labeled and radio-labeled phospholipids, neutral lipids and CDP-diacylglycerols were shown to occur between microsomes and mitochondria of guinea pig liver. A similar transfer of phosphatidic acid was reported between microsomes and mitochondria of rat liver [28]. In view of the wide spectrum of lipid transfer activity we believe that these lipid transfers are mediated by the nonspecific lipid transfer protein bound to mitochondria and possibly microsomes [25,29,30].

Under the conditions of lipid transfer assays nonspecific lipid transfer protein most likely remains bound to the mitochondrial membrane. This then raises the question as to how nonspecific lipid transfer protein mediates lipid transfer between membranes. Stuhne-Sekalec and Stanacev have concluded from their studies that the 'spontaneous' transfer of lipids from microsomal to mitochondrial membranes requires a close contact between these membranes [11]. In accordance with this conclusion we have postulated that nonspecific lipid transfer protein may be instrumental in this contact process, thereby allowing lipids to redistribute between membranes [31]. Recently we have shown that nonspecific lipid transfer protein has an essential SH-group. Modification of this group by thiol reagents blocks the transfer activity [31]. Treatment of intact mitochondria with *N*-ethylmaleimide and mersalyl greatly reduces the lipid transfer (Ref. 12, Fig. 1), strongly suggesting that nonspecific lipid transfer protein bound to the membrane becomes inactivated. It remains to

be established in what way the free SH-group on nonspecific lipid transfer protein is involved in its mode of action.

Mitochondria have a very limited capacity for phospholipid synthesis and are dependent on the endoplasmic reticulum for the bulk of their lipid constituents. It is possible that nonspecific lipid transfer protein is involved in this intracellular lipid transfer to the mitochondria. Similarly, Scallen and co-workers have provided evidence that nonspecific lipid transfer protein/(sterol carrier protein 2) mediates the intracellular transfer of cholesterol to mitochondria in steroidogenic tissues, thereby playing a role in the conversion of cholesterol to pregnenolone [32]. Very recently Van Noort et al. [33] provided evidence for a selective occurrence of nonspecific lipid transfer protein in the Leydig cells of rat testis, confirming its proposed role in cholesterol metabolism.

The presence of nonspecific lipid transfer protein in subcellular membrane fractions different from mitochondria has been demonstrated [22]. In a recent study Seltman et al. [34] investigated the microsomal conversion of cholesterol into bile acids in rat liver. Addition of specific antibodies to nonspecific lipid transfer protein/(sterol carrier protein 2) inhibited this conversion, strongly suggesting that nonspecific lipid transfer protein/(sterol carrier protein 2) bound to microsomes was involved in this process. On the other hand 70% of nonspecific lipid transfer protein is found in the supernatant [22]. Similarly there is some evidence that nonspecific lipid transfer protein can be secreted into the medium by hepatoma cells in culture [35]. In view of these observations we believe that one has to be careful in assigning a specific physiological function to this protein.

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