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Porcine aortic endothelial cell membranes contain a LPAF : CoA-independent transacylase

Gerard J. Jansen, Carole R. Pritzker and Daniel Deykin

Department of Medicine and Biochemistry, Boston University School of Medicine, Boston, MA (USA) and Boston Veterans Administration Medical Center, Boston, MA (USA)

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Membranes isolated from porcine aortic endothelial cells (PAEC) contain a CoA-independent transacylase enzyme (CoA-IT). CoA-IT, an integral membrane protein, transfers an acyl moiety to added [^{3}H]alkylhydroxyglycerophosphocholine (LPAF) to generate [^{3}H]alkylacylglycerophosphocholine (alkylacyl-GPC). This enzyme exhibits an apparent K_m of 0.7 μM and a V_{max} of 0.8 nmol/min per mg for the transfer of an acyl group to added [^{3}H]LPAF. The addition of the nonionic detergent Triton X-100 (TX-100) (0.5 mg/ml), the sulfhydryl reagents *N*-ethylmaleimide (NEM) (200 μM) or thimerosal (200 μM), or pre-incubating the membranes at 95°C for 10 min all decreased LPAF:CoA-IT activity by more than 95%. The inhibitory action of NEM or thimerosal suggests that sulfhydryl group(s) are involved in or are close to the catalytic site of LPAF:CoA-IT.

Introduction

CoA-independent transacylase (CoA-IT) is an integral membrane protein which catalyses the direct transfer of an acyl group, usually arachidonic acid (AA), from diradylglycerophosphocholine (diradyl-GPC) to added lysophospholipid. CoA-IT has been found in a variety of tissues [1–13] and appears to play a key role in membrane phospholipid remodeling, transferring arachidonic acid from diacyl-GPC to both alkenyllyso-GPE and alkyllyso-GPE [1,6]. In rat platelets pre-labelled with [^{14}C]AA, there is specific CoA-IT transfer of arachidonate from diacyl-GPC to diacyl-GPE in both stimulated [14] and resting platelets [15]. In both bovine aortic endothelial cells [16] and porcine aortic endothelial cells (PAEC's) [17], given a 2 h [^{14}C]AA pulse, the content of [^{14}C]AA-PE increased to the same extent that the content of [^{14}C]AA-PC decreased over the following 24 h chase.

While a variety of lysophospholipids are used to assay CoA-IT activity, the acyl acceptor [^{3}H]alkyllysoglycerophosphocholine ([^{3}H]LPAF) is of particular interest. LPAF is the precursor for platelet activating factor (PAF, alkylacylglycerophosphocholine) which has marked biological activity. A variety of tissues including rabbit endothelial cells [18] and cultured human endothelial cells [19] release PAF after stimulation by thrombin. However, due to its high biological activity, released PAF is rapidly deacylated by acetylhydrolyase [20], producing the biologically inactive precursor LPAF.

Until recently the singular pathway of LPAF generation was considered to be an activation cascade culminating in the activation of phospholipase A_2 (PLA_2) to generate LPAF [21]. Using membranes pre-labelled with [^{3}H]alkylacyl-GPC, Uemura et al. [13] reported that LPAF was *generated* by CoA-independent transacylase (CoA-IT) when it utilized [^{3}H]alkylacyl-GPC as the acyl donor and LPPE as the acyl acceptor. This finding expands the role of transacylase in membrane phospholipid remodeling to include the generation of specific lysophospholipids. It would appear to demonstrate that CoA-IT is able to utilize a variety of both acyl donors and acyl acceptors. However, precise elucidation of the substrate specificity requires purified CoA-IT and may reveal the existence of more than one CoA-independent transacylase in any tissue.

Correspondence to: G.J. Jansen, Department of Hemostasis Research, Boston Veterans Administration Medical Center, 150 South Huntington Avenue, Boston, MA 02130, USA.

Abbreviations: LPAF, alkyllysoglycerophosphocholine; alkylacyl-GPC, alkylacylglycerophosphocholine; CoA-IT, coenzyme A-independent transacylase; AA, arachidonic acid; LCAT, phosphatidylcholine:sterol *O*-acyltransferase; TX-100, Triton X-100; NEM, *N*-ethylmaleimide; Brij-35, polyoxyethylene 23 lauryl ether; OG, n-octyl β-D-glucopyranoside; mV, millivolts; GPE, alkylacylglycerophosphoethanolamine; LPPE, alkenyllysoglycerophosphoethanolamine; LPPC, alkenyllysoglycerophosphocholine; PAEC, porcine aortic endothelial cells.

We now report that the membranes isolated from porcine aortic endothelial cells contain a CoA-IT which transfers an acyl moiety to added [^{3}H]LPAF to produce [^{3}H]alkylacyl-GPC. The PAEC LPAF:CoA-IT described exhibits both the kinetics (apparent V_{max} and K_m) and the sensitivity to sulfhydryl reagents consistent with those reported for other CoA-independent transacylases [1,6].

Methods

Chemicals

Bovine serum albumin (BSA) (fatty-acid free), CoA and all chemicals were obtained from Sigma (St. Louis, MO). All solvents used were spectrograde quality from Fisher (NY). Lipids were purchased from Avanti Polar Lipids (Pelham, AL) and were > 99% pure as determined by HPLC-FITC and TLC (two solvents, both at 65 : 25 : 4, chloroform/methanol/water and chloroform/methanol/concentrated ammonium hydroxide using Whatman K6 TLC plates). The radioisotope [^{3}H]LPAF (30–60 Ci/mmol) was obtained from New England Nuclear (Boston, MA).

Porcine aortic cell isolation and culture

Freshly isolated porcine thoracic aortas were obtained from a local abattoir. The endothelial cells were isolated and grown in tissue culture as previously described [22].

Plasma membrane isolation

PAEC cultures in the logarithmic phase of growth (75% + confluent) were rinsed three times with 37°C, sterile buffered Hanks' balanced salt solution (pH 7.4; Sigma, St. Louis, MO). 4 ml of buffer A (120 mM NaCl, 30 mM Tris, 5 mM EGTA and 0.1 mM β-mer-

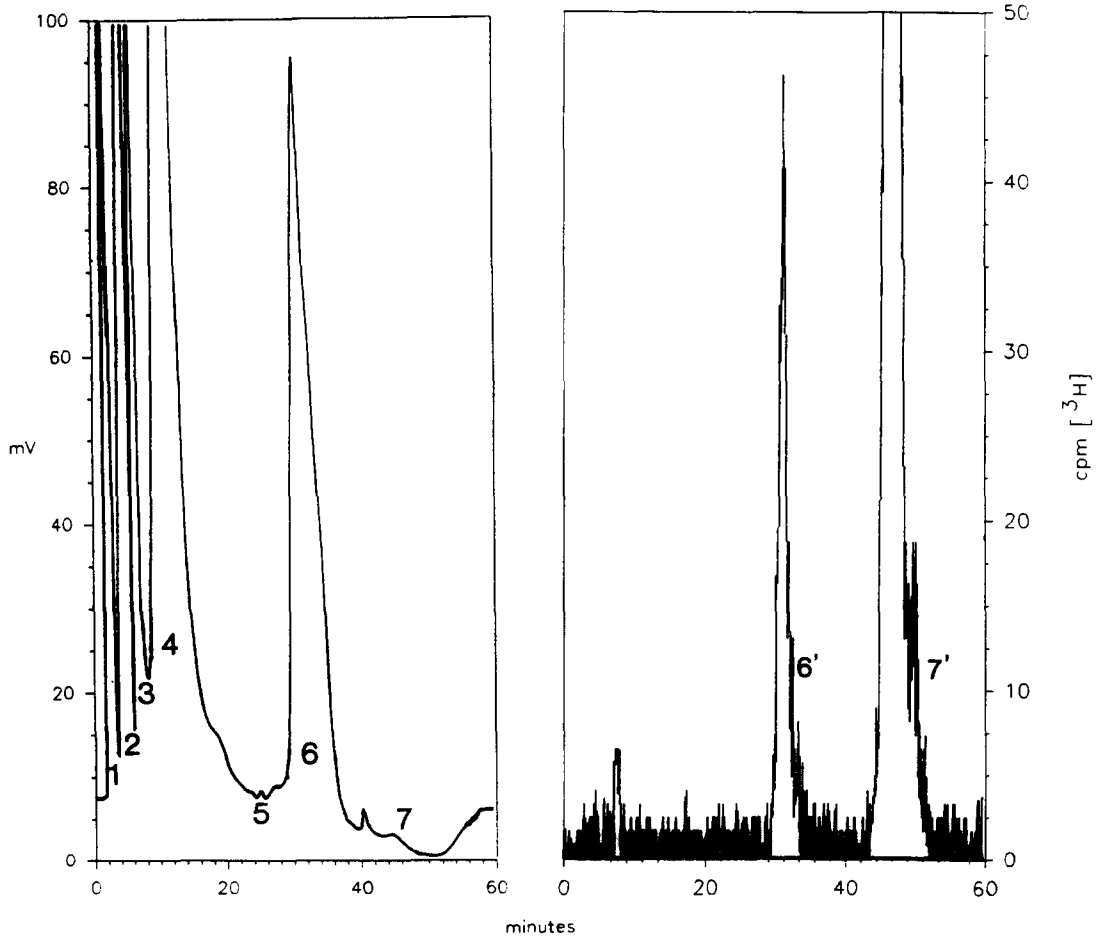

Fig. 1. Separation and determination of the radioactivity of extracted lipids. The plasma membranes (50 μg) containing LPAF:CoA-independent transacylase were isolated from porcine aortic endothelial cells and incubated with [^{3}H]alkyllysoglycerophosphocholine at 37°C. After 1 min the lipids were extracted and then separated using HPLC and counted for radioactivity using a Radiomatic Online β-counter. The lipids were identified by comparison to standards. 1, injection peak; 2, neutral lipids; 3, diradylglycerophosphoinositol/serine; 4, diradylglycerophosphoethanolamine; 5, alk-1'-enelysoglycerophosphoethanolamine; 6, diradylglycerophosphocholine; 7, radyllysoglycerophosphocholine. The radioactive lipids are 6', [^{3}H]alkylradylglycerophosphocholine; 7' [^{3}H]alkyllysoglycerophosphocholine ([^{3}H]-LPAF)].

captoethanol, pH 7.5) at 37°C containing a cocktail of inhibitors (final concentration leupeptin, 0.1 μg/ml; pepstatin, 0.5 μg/ml; aprotinin, 0.1 μg/ml) was added to each 100 mm^2 plate, and the cells removed with a rubber scraper. The plate was then rinsed with an additional 2 ml of buffer A which was added to the scraped cells. The PAEC's were suspended using a pre-cooled (4°C) Kontes Potter-Elvehjem Tissue grinder (loose fit) and then sonicated on ice (4°C) at 50 watts using a sonicator with a microprobe (Heat-System Ultrasonic) for 2 × 15 s. The PAEC homogenate was diluted to 30 ml per 100 mm^2 dish, centrifuged at 10 000 × g for 20 min and the supernatant then centrifuged at 100 000 × g for 45 min to produce a 'plasma membrane rich' pellet [26]. Plasma membranes were resuspended to a protein concentration [29] of 10 mg/ml in buffer A and snap frozen in solid CO_2/ethanol and stored at −70°C. The plasma membrane preparation was assayed for the presence of two marker enzymes, glucose-6-phosphatase [32] an endoplasmic reticulum marker and for alkaline phosphatase, a plasma membrane marker enzyme [33].

Incubation of membranes with substrates, separation and identification of products

The substrate LPAF was diluted to the required concentration, and [^{3}H]LPAF was added to produce a specific activity of 100 000 dpm per pmol. The [^{3}H]LPAF was pipetted into ethanol rinsed 20 ml glass Kimax tubes and dried under N_2. Immediately after drying, buffer A containing 0.1% BSA (final concentration) was added to the Kimax tubes and incubated at 37°C for 10 min. To begin the reaction, 50 μg of PAEC membrane protein was added (final volume was 200 μl) and the assay cocktail incubated in a shaking water-bath at 37°C. To halt the reaction, 5 ml of ice-cold chloroform/methanol (2:1, v/v) was added and after vigorous vortexing was left for 10 min at room temperature. The control for each experiment was an equivalent amount of membrane protein heated at 95°C for 10 min. After addition of 0.58% NaCl to generate two phases, the bottom lipid containing organic layer was removed, and then the residue re-extracted [23]. The lipid-containing lower organic layers were combined and evaporated under N_2. Dried lipids were immediately re-dissolved with 250 μl of solvent and injected onto a Spherisorb normal phase column (250 × 4.6 mm; S5W), and eluted with an acetonitrile/water (80:20, v/v) gradient [24,25]. The radioactivity of the lipids, after absorbance monitoring, was continuously determined using a Radiomatic FLO-ONE/A140 online β counter, with a FLO-SCINT II:HPLC eluant ratio of 3:1 (ml/min). Identification of the fractions was achieved by reference to the retention times of both single and mixed standards of both radio-labeled and unlabeled phospholipids.

Results

The 'plasma membrane' pellet was isolated from sonicated PAEC'S using a common protocol [26]. We found that this pellet contained 2.2 IU/min per mg of alkaline phosphatase activity and 0.004 μg phosphorus/min per mg glucose-6-phosphatase (G6P) activity, and therefore our plasma membranes contain a small amount of endoplasmic reticulum membranes.

A typical separation and on-line determination of lipid radioactivity is shown in Fig. 1. The peaks of both tracings were identified by comparison to pure lipids and to pure radio-labeled [^{3}H]LPAF and [^{3}H]alkylacyl-GPC. The transacylation of added [^{3}H]LPAF to [^{3}H]alkylacyl-GPC by LPAF:CoA-IT over a period of 30 min is shown in Fig. 2. Initially there is rapid activity so that by 6 min more than 90% of the maximal transacylation had been achieved. The maximal activity of LPAF:CoA-IT with increasing membrane protein is shown in Fig. 3. At a substrate concentration of 200 μM, there is increasing CoA-IT activity with increasing membrane protein and more than 90% maximal LPAF:CoA-IT occurs with just 50 μg of membrane protein. From these data we determined that the optimal conditions for further LPAF:CoA-IT studies were 50 μg membrane protein in 200 μl buffer A incubated for 10 min at 37°C. The Lineweaver-Burk plot of PAEC CoA-IT activity (Fig. 4) yields an apparent K_m of 0.72 μM and a V_{max} of 0.8 nmol/min per mg protein

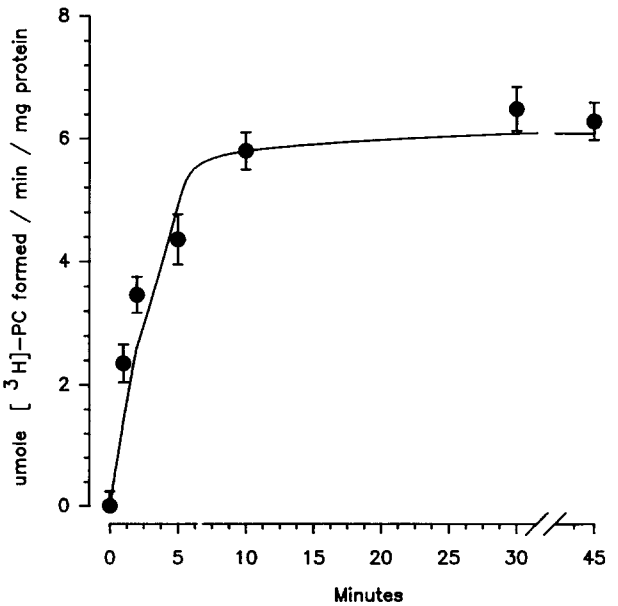

Fig. 2. Time course of LPAF:CoA-IT activity. Plasma membranes (50 μg) isolated from porcine aortic endothelial cells were incubated with 100 μM [^{3}H]alkyllysoglycerophosphocholine in 200 μl of buffer A containing 0.1% BSA (final) for 10 min at 37°C. The lipids were extracted, separated using HPLC and counted for radioactivity. Each data point is the mean ± S.D. of three experiments in which each point was assayed in duplicate.

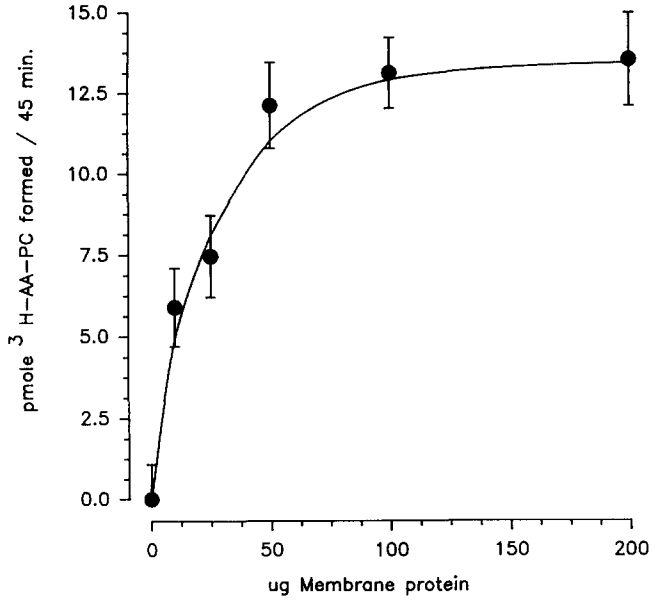

Fig. 3. Activity of LPAF:CoA-IT in increasing quantities of plasma membrane. Increasing amounts of membranes were incubated with 100 μM of [^{3}H]alkyllysoglycerophosphocholine in a volume of 200 μl of buffer A containing 0.1% BSA. After incubating 10 min at 37°C, the lipids were extracted and then separated using HPLC and the fractions counted for radioactivity. Each point is the mean ± S.D. of three experiments carried out in duplicate.

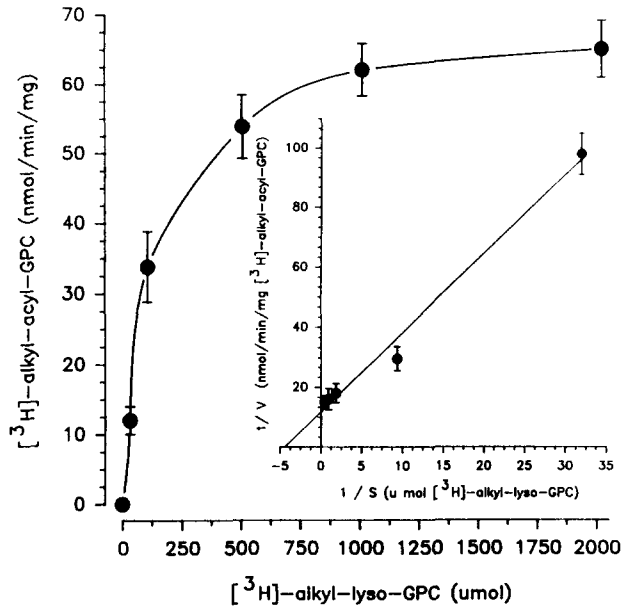

Fig. 4. Determination of the K_m and V_{max} of LPAF:CoA-IT in PAEC plasma membranes. The plasma membranes (50 μg) were incubated with increasing quantities of [^{3}H]alkyllysoglycerophosphocholine and 0.1% BSA in a volume of 200 μl of buffer A. After a 10 min incubation at 37°C in a shaking waterbath, LPAF:CoA-IT activity was stopped by lipid extraction. (Inset) Lineweaver-Burk plot of the data to determine apparent K_m and V_{max}. Each point shown is the mean ± S.D. of at least three experiments carried out in duplicate.

TABLE I

Effect of various inhibitors on PAEC LPAF transacylase activity

The PAEC membranes were pre-incubated with the inhibitor for 10 min at 37°C and then assayed for LPAF transacylase activity as described in Methods. Results are the means ± S.D. of two determinations carried out in duplicate.

Inhibitor		%LPAF:CoA-IT activity
None, control		100
NEM	50 μM	40 ± 6
	200 μM	0 [a]
Thimerosal [b]	0.1 μM	85 ± 6
	1.0 μM	78 ± 5
	10 μM	60 ± 5
	50 μM	32 ± 4
	200 μM	0 [a]
Heat	95°C:10 min	0 [a]
	45°C:10 min	25 ± 5
TX-100	0.1 mg/ml	75 ± 8
	0.5 mg/ml	0 [a]
Brij-45	0.5 mg/ml	95 ± 5
Octyl glucoside	0.5 mg/ml	85 ± 6

[a] No LPAF:transacylase activity was detected.

[b] Estimate of the IC_{50} for CoA-IT *in the membranes* is 15 μM thimerosal per 50 μg of total plasma membrane protein.

for the transacylation of [^{3}H]LPAF to [^{3}H]alkylacyl-GPC.

The effect of various transacylase inhibitors [25] on LPAF:CoA-IT activity is presented in Table I. The sulfhydryl alkylating agents *N*-ethylmaleimide (200 μM) and thimerosal (200 μM) totally inhibited LPAF:CoA-IT, indicating that SH-groups are in or close to the active site. The thimerosal data indicates that the IC_{50} of LPAF:CoA-IT *in the plasma membrane* is 15 μM. Pre-heating the membranes to 45°C for 10 min reduces LPAF:CoA-IT activity by 75%, and no activity is detected after pre-incubation at 95°C for 10 min. The nonionic detergents tested had a varied effect on activity, with the addition of 0.1 mg/ml TX-100 reducing activity by 25%, and 0.5 mg/ml TX-100 completely abolishing activity. This is in marked contrast to the effects of polyoxyethylene 23 lauryl ether (Brij-35) or n-octyl β-D-glucopyranoside (OG), both of which increased CoA-IT activity slightly at the low concentration of 0.1 mg/ml and caused no change in the level of LPAF:CoA-IT activity at 0.5 mg/ml. Additions of OG above 1.0 mg/ml caused a gradual decline in LPAF:CoA-IT activity.

Discussion

Data indicate that membranes obtained from porcine aortic endothelial cells contain a LPAF:CoA-IT, adding to the growing list of tissues where this enzyme has been found [1–11]. Our procedure of cell disruption by sonication followed by sequential centrif-

TABLE II

Kinetic parameters of CoA-independent transacylases

Source	Acceptor	Apparent K_m (μM)	V_{max} (nmol/min per mg)	Ref.
Endothelial	LPAF	0.7	0.83	this paper
Platelets	LPPE	20	1.9	1
(human)	LPAF	12	0.87	2
Monocytes				
(U937)	LPAf	0.4	0.1	6
Macrophages	LPAF	1.1	3.2	31
Microsomes				
rat liver	retinol	2.0	0.047	5
rat mammary	retinol	2.0	0.008	

ugation yielded a plasma membrane enriched fraction which also contained some microsomes from both smooth and rough endoplasmic reticulum and Golgi [26]. Initially we found that the K_m varied when the primary centrifugation step was decreased in either time or *g* forces, probably due to increased amounts of other membranes [26]. These results suggest that LPAF:CoA-IT is not uniformly distributed in cellular membranes. The K_m values reported for LPAF:CoA-IT's from other tissues range from 12 μM for human platelet membranes [2] to 0.4 μM for U937 monocytic cells [6] (Table II). All the K_m values for transacylases have been determined with the enzyme still in the native membrane, so they will likely decrease when redetermined using purified transacylase protein. The PAEC V_{max} of 0.8 nmol falls within the range of 3.2 μmol for macrophages [12] to 0.1 μmol for monocytes (U937) [6].

The differing effect of the nonionic detergents TX-100 and OG on PAEC CoA-IT is very similar to those reported by Kramer [1] for the LPPE:CoA-IT in human platelet membranes. A low concentration (0.1 mg/ml) of TX-100 had a marked inhibitory effect on LPPE:CoA-IT, but OG at 0.1 mg/ml stimulated activity. Increasing concentrations of OG (above 1.0 mg/ml) caused decreasing CoA-IT activity, probably indicating the gradual disruption of the membrane structure.

Inhibition of LPAF:CoA-IT activity by the sulfhydryl reagents (Table I) would suggest that sulfhydryl groups take an active role in the CoA-IT catalytic mechanism. A model proposed for the catalytic mechanism of phosphatidylcholine:sterol *O*-acyltransferase (LCAT) enzyme by Jauhianinen et al. [27] was disproved by site-directed mutagenesis in which the two active-site cysteines were replaced. The mutant LCAT retained catalytic activity but was only partially inhibited by 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) [28]. From their data Francone and Fielding [28] proposed that the DTNB inhibition of LCAT was due to DTNB binding to cysteines and sterically inhibiting substrate binding. The inhibition of LPAF:CoA-IT by thimerosal and NEM indicates that LPAF:CoA-IT has sulfhydryl groups in the substrate binding pocket.

The role played by CoA-independent transacylase in membrane phospholipid remodeling is expanding. A recent paper by Uemura et al, [13] has demonstrated that in the presence of [^{3}H]alkylacyl-GPC and the acyl acceptor LPPE, CoA-IT would *generate* [^{3}H]-LPAF. It was previously considered that CoA-IT's only function was the transfer of an *sn*-2 acyl group, usually arachidonate, from the diacyl-GPC to either alkyllyso-GPE or alkenyllyso-GPE. This result suggests that, at least under experimental conditions, CoA-IT will transfer an acyl moiety from either diacyl-GPC or alkylacyl-GPC to added acceptor. In addition to two acyl donors, the range of acyl acceptors that CoA-IT utilizes: LPAF; LPPE; LPPC and retinol suggests an indiscriminate binding site. However, we have found that human platelet membranes appear to contain two transacylases which are specific for the acyl acceptor, a LPAF:CoA-IT and a LPPE:CoA-IT (Jansen, G.J., Pritzker, C.R. and Deykin, D., submitted). Of necessity all substrate studies have examined the substrate preferences with the CoA-IT remaining within its native membrane. The native membrane contains a wide range of both phospholipids and numerous other proteins. Therefore elucidation of the precise substrate preferences and deduction of the specific role played by CoA-IT in membrane phospholipid remodeling will likely require studies using purified CoA-IT reconstituted into liposomes containing specific phospholipids.

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