Enzyme-coupled assays for flip-flop of acyl-Coenzyme A in liposomes

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Acyl-Coenzyme A is made in the cytosol. Certain enzymes using acyl-CoA seem to operate in the lumen of the ER but no corresponding flipfasses for acyl-CoA or an activated acyl have been described. In order to test the ability of purified candidate flipases to operate the transport of acyl-CoA through lipid bilayers in vitro we developed three enzyme-coupled assays using large unilamellar vesicles (LUVs) obtained by detergent removal. The first assay uses liposomes encapsulating a water-soluble acyl-CoA:glycerol-3-phosphate acyl transferase plus glycerol-3-phosphate (G3P). It measures formation of [3H]lyso-phosphatidic acid inside liposomes after [3H]palmitoyl-CoA has been added from outside. Two other tests use empty liposomes containing [3H]palmitoyl-CoA in the inner membrane leaflet, to which either soluble acyl-CoA:glycerol-3-phosphate acyl transferase plus glycerol-3-phosphate or alkaline phosphatase are added from outside. Here one can follow the appearance of [3H]lyso-phosphatidic acid or of dephosphorylated [3H]acyl-CoA, respectively, both being made outside the liposomes. Although the liposomes may retain small amounts of detergent, all these tests show that palmitoyl-CoA crosses the lipid bilayer only very slowly and that the lipid composition of liposomes barely affects the flip-flop rate. Thus, palmitoyl-CoA cannot cross the membrane spontaneously implying that in vivo some transport mechanism is required.

1. Introduction

Living organisms are surrounded by lipid-containing membranes, which in eukaryotes are mainly made of glycerophospholipids, sphingolipids and sterols. Glycerophospholipids are made from phosphatidic acid (PA), a central metabolite, which in eukaryotes is generated through acyl-Coenzyme A (acyl-CoA) dependent reactions from glycerol-3-phosphate (G3P) [1]. G3P is first acylated in sn-1 by a glycerol-3-phosphate acyltransferase (GPAT) and then in sn-2 by a 1-acylglycerol-3-phosphate acyltransferase (AGPAT). The numerous eukaryotic GPATs and AGPATs mostly belong to either one or the other of two gene families, characterized by the pfam01553 and pfam3062 motifs, respectively. GPATs and AGPATs found in animals and yeast are transmembrane proteins, while chloroplasts of some plant species contain soluble luminal GPAT homologs, one of which, the one of squash (Cucurbita moschata), has been crystallized [2,3]. The pfam3062 motif is characteristic of membrane bound O-acyltransferases (MOBATS), a huge superfamily of ER proteins with multiple transmembrane domains. A strictly conserved His residue in the pfam3062 motif is considered to be the critical active site residue [4] and, in eukaryotes, to reside in the ER lumen. The luminal location of the active site of MOBAT proteins is not only supported by biochemical investigations of the topology of the conserved His residue [5–9], but also by the fact that some MOBATS acylate ER luminal secretory proteins such as Hedgehog, Wnt, Ghrelin and yeast GPI anchored proteins [10–14]. Also, lyso-GPI anchors are acylated in the lumen of the mammalian Golgi by PGAP2 [15] and the GPI biosynthetic intermediate phosphatidylinositol-glucosamine (PI-GlcN) is acylated on the inositol moiety in the ER lumen by Gwt1 [16,17]. PGAP2 and Gwt1 do not belong to the above mentioned pfam motif families. Acyl-CoA dependent acylation reactions in the lumen of the secretory apparatus imply that acyl-CoA or the acyl group of acyl-CoA may have to be transported through organelar membranes.

Best characterized are the mechanisms used to bring fatty acids into mitochondria or peroxisomes for β-oxidation. In mitochondria, acyls are transferred from CoA to carnitine and transported as acyl-carnitine through the inner membrane, to then be transferred again onto CoA in the matrix. There also is compelling evidence for the role of two ATP-driven ABC half transporters, Pxa1 and Pxa2 required for the import of long chain fatty acids into yeast peroxisomes and similar transporters exist in mammals and plants. However, it still is not totally clear if Pxa1/2 act as bona fide acyl-CoA flipases or only transport the fatty
acid moiety [18–20]. With regard to the ER, no acyl-CoA flippases have been identified yet.

Here we undertook to set up assays that may be used to measure the activity of acyl-CoA transporters after their reconstitution into large unilamellar vesicles (LUVs).

2. Materials and methods

Sources of materials can be found in the Supplementary materials.

2.1. Encapsulation of sqGPAT and G3P into large unilamellar vesicles (LUVs)

Liposomes with encapsulated sqGPAT and G3P were formed by detergent removal using Bio-bead SM-2 adsorption [21]. In the standard protocol, 2.5 mg of lipids was dried under vacuum for 4 h at 37 °C. 1 ml of buffer D (20 mM potassium phosphate buffer pH 7.0, 50 mM NaCl) containing 1% of detergent (C12E9, octyl-β-D-glucopyranoside (OGP) or TX-100), 1 mM G3P and 15 nmol (657 μg) sqGPAT was added to the dried lipids; the tube was shaken gently for 30 min on a rotating wheel. Thereafter, detergent was removed using SM-2 beads adapting the protocol of [22]; For C12E9 or OGP, the solution was incubated on an end-over-end rotator with 150 mg (wet weight) of SM-2 beads for 2 h. Then, 300 μg of fresh beads was added and shaking was continued for 12 h (overnight). Thereafter, the liposome solution (without beads) was transferred to a new tube containing 500 mg of fresh SM-2 beads and incubated for an additional 3 h. All steps were performed at 4 °C unless stated otherwise. TX-100 was removed as above but using 150 mg of beads, incubating 3 h, adding 250 mg of fresh beads and incubating another 3 h. Liposomes were then transferred to fresh 300 mg beads for 12 h, and finally to fresh 300 mg beads for 2 h. This procedure results in the formation of LUVs, the size of which depends on the detergent: C12E8 yields vesicle populations with a modal diameter of 90 nm, TX-100 of 240 nm and OGP of >300 nm [23]. Unless indicated otherwise, OGP was used, but the same results were obtained with liposomes made using C12E9 or TX-100.

To digest non-encapsulated sqGPAT potentially sticking to the outside of the LUVs, the liposomes were incubated with 50 μg (1.73 μM) proteinase K at 4 °C for 1 h. Proteinase K was inactivated by adding PMSF and AEBSF to 2 and 0.5 mM and further incubating for 1 h at 4 °C. Inactivated proteinase K and peptides were then removed by dialysis (cut-off 100 kDa) against 100 ml buffer D containing 1 mM G3P at 4 °C for 15 h. Dialysis had no effect on the results and was omitted in later preparations.) In all experiments shown here we used proteinase K to shave the liposomes, but trypsin achieved the same result (Fig. S1). Lipid loss was estimated by quantifying phosphatidylcholine (PC) shave the liposomes, but trypsin achieved the same result (Fig. S1). Lipid loss was estimated by quantifying phosphatidylcholine (PC) that the enzyme can utilize acyl-CoA presented by LUVs if the surface concentration of palmitoyl-CoA is above CMC for the liposomes. The concentration in the lumen was assumed to be 1 mM, as all buffers used throughout contained 1 mM G3P.

2.2. LPA generation in liposomes encapsulating sqGPAT and G3P

In most experiments, [3H]palmitoyl-CoA (1 μg, 17 pmol) was added to liposomes equivalent to 100 nmol of palmityl-oleoyl-PC/cholesterol (POPC/Cho; 70:30 mol%) encapsulating sqGPAT/G3P in a final volume of 100 μl buffer D supplemented with 1 mM G3P. The reaction was incubated up to 60 min at RT. As a positive control, liposomes were preincubated for 10 min at RT with perfringolysin O (POFO) (0.7 nmol) in buffer D supplemented with 1 mM G3P and 1 mM DTT (to maintain POFO activity). Alternatively, detergents (TX-100 or OGP) were added to the assays in a 10-fold molar excess over lipids. Reactions were stopped by the addition of 2.5 μl HCl (1 M) to reach a final pH < 4 (in order to increase solubility of LPA in butanol) and vigorous vortexing. Lipids were extracted 3 times by adding 350 μl butanol to 100 μl of reaction solution (aqueous phase), vortexing and subsequently removing the butanol phase with a pipette. The pooled butanol fractions were dried under vacuum in a rotary evaporator and resuspended in 30 μl of solvent. Samples were applied to TLC plate (silica gel 60) and dried for 60 min under laminar airflow. Samples were analyzed by TLC using the solvent system chloroform/methanol/0.25% KCl (55:45:5). Plates were analyzed by phosphorimaging and radioscopying (Berthold Services GmbH, Switzerland). All quantifications of bands were obtained through radioscopying.

2.3. Incorporation of [3H]palmitoyl-CoA into liposomes

Normally, 2.5 mg of lipids together with [3H]palmitoyl-CoA in organic solvent were mixed and dried under vacuum in the rotary evaporator at 20 °C for 4 h. Buffer D containing 1% of detergent (C12E9, OGP or TX-100) was added and liposomes were prepared as described above. The resulting liposomes containing [3H]palmitoyl-CoA were stored at 4 °C and were usually used within 6 h. Palmitoyl-CoA has detergent-like properties and is also partially removed by the SM-2 beads, but the final lipid preparation usually still contained 3–10% of the radioactivity added in the beginning.

2.4. Phosphatase treatment of liposomes containing [3H]palmitoyl-CoA

Liposomes containing [3H]palmitoyl-CoA were subjected to bovine intestinal alkaline phosphatase (AP) treatment: Typically, 30 μl of [3H]palmitoyl-CoA containing liposomes (100 nmol lipids, 17 pmol [3H]palmitoyl-CoA) was treated with 10 U of AP in a final volume of 100 μl buffer D. In the case of measuring the production of LPA, 60 pmol (2.6 μg) sqGPAT and 100 nmol G3P were added to the liposomes in 100 μl of buffer D. Reactions took place at RT. Reactions were stopped by addition of 2.5 μl HCl (1 M) and lipids extracted and analyzed as described above.

All other methods used are described in the Supplementary materials section.

3. Results

3.1. LUVs can replace bovine serum albumin in the sqGPAT activity assay

As reported before for another soluble GPAT [25], LPA is formed by sqGPAT only in the presence of bovine serum albumin (BSA), which seems to be required for making acyl-CoA more accessible for sqGPAT (Fig. S2A). Yet we found that large unilamellar vesicles (LUVs) could replace BSA in this function if they were added in sufficient quantity (Fig. S2B). As the concentration of palmitoyl-CoA (10 μM) in the assays was below its critical micelle concentration (CMC) of 75 μM [26], data indicate that acyl-CoA monomers are not recognized by sqGPAT, but that the enzyme can utilize acyl-CoA presented by LUVs if the surface density of acyl-CoA falls below a certain critical value, which seems to be around 10–20 mol% (Fig. S2B). On the other hand, sqGPAT did not work in the presence of detergents such as OGP, TX-100, or dodecylmaltoside (tested at 2.5 mM, i.e. above CMC for the last two) (not shown). Also, when added to assays containing BSA, these same detergents inhibited sqGPAT in a concentration dependent manner (not shown).

3.2. Assays using liposomes encapsulating squash GPAT together with G3P

Palmitoyl-CoA can be regarded as a detergent and we wondered at what concentration it would dissolve liposomes. For this we produced LUVs filled with calcein, a fluorophore, which is self-quenching at concentrations of above 50 mM [27]. LUVs made by extrusion, i.e. without detergent, could not be lysed even at palmitoyl-CoA:lipid ratios of 1:6.1, although small transient leakages causing around 1% leakage of total encapsulated calcein were occasionally observed upon addition of acyl-CoA (Fig. S3). However, in view of reconstituting proteins into
the liposome bilayer, we wanted to make LUVs from detergent lipid mixtures and we observed in many experiments that μM concentrations of acyl-CoA could lyse part of LUVs generated by the detergent removal method (not shown). Moreover, sub-solubilizing concentrations of detergents can accelerate the flip-flop of fluorescently labeled PC analogs in membranes [28]. We therefore opted for the use of radiolabeled [3H]palmitoyl-CoA, which allowed detecting GPAT activity at [3H]palmitoyl-CoA/lipid ratios of 1:6000 or less. For our typical assay, [3H]palmitoyl-CoA was added to palmitoyl-oleoyl-PC/cholesterol (POPC/Cho, 70:30 mol%) liposomes containing encapsulated sqGPAT and G3P (Fig. 1).

To avoid any loss of G3P from liposomes through slow leakage, 1 mM G3P was present throughout the preparation of liposomes and was also added at the moment of the assay. For a positive control, liposomes were preincubated with the pore forming toxin perfringolysin O (PFO). PFO pores are big enough to allow not only entrance of acyl-CoA but also exit of sqG3P so that in this condition sqGPAT was able to access [3H]palmitoyl-CoA from both sides of the liposomal membrane [29]. As can be seen in Fig. 1, penetration of acyl-CoA into liposomes was clearly rate-limiting in the assay, as the rate of LPA formation was at least 10 fold lower with intact liposomes (lanes 1–4) than when liposomes were opened using PFO (lanes 5–8). This experiment was repeated 4 times in slightly different conditions and yielding similar results in that only a few percent of radioactivity were transformed into LPA in 15 min in non-permeabilized liposomes (not shown). According to literature, the flip-flop rate of acyl-CoA in liposomes made under detergent-free conditions is even slower [30]. The slow synthesis of LPA in our assays could be due either to incomplete detergent removal by SM-2 beads or to small amounts of sqG3P sticking to the outside of liposomes and having survived proteinase K digestion. We however are confident that the small amounts of LPA generated during the assay cannot cause any disruption of the liposomal bilayer. While LPA is a potential detergent, the synthesis of each LPA molecule causes the disappearance of an acyl-CoA, which can have detergent activity in its own right. Thus, the ratio [acyl-CoA + LPA]/[lipid] remains constant at 1:6000 during the assay.

![Image](http://doc.rero.ch)

**Fig. 1.** Addition of [3H]palmitoyl-CoA to liposomes containing encapsulated sqGPAT and G3P results in formation of LPA. 10 pmol [3H]palmitoyl-CoA was added to POPC/Cho (70:30 mol%) liposomes containing encapsulated sqGPAT and G3P (final: 100 nmol lipids, 1.3 μg sqGPAT, 100 nmol G3P) in a final volume of 100 μl. As a positive control, liposomes were preincubated with PFO. Samples were incubated from 1 to 15 min, lipids were extracted, separated by TLC, and visualized by using a Phosphorimager. In lane 9, sqG3P was omitted. Radioactivity in LPA is given in the last row as % of the sum of radioactivity present at the origin plus in LPA, whereas the bands at the top were not taken into account, because they were present in the [3H]palmitoyl-CoA preparation added to the assay.

3.3. Assays avoiding the need for protease digestion

The above assay is amenable to testing the flip-flop activity of pore-forming peptides and drugs, but it utilizes proteases to remove non-encapsulated sqGPAT. We have not investigated if non-encapsulated sqGPAT can be removed by mere dialysis because we surmised that some sqGPAT would be passively adsorbed to the liposomal surface and therefore routinely used protease treatment. Therefore, the above-described protocol cannot be utilized to test the flip-flop activity of proteins, since the protease treatment of course also may activate a reconstituted potential transporter. To assay flip-flop activity of membrane proteins, two different types of protease-free assays were created. For both assays we incorporated [3H]palmitoyl-CoA into the liposomes. Such liposomes could be obtained by adding SM-2 beads to lipid/[3H]palmitoyl-CoA/detergent mixtures in order to remove detergent. Because of its detergent-like character, about 90% of the [3H]palmitoyl-CoA added at the beginning was also removed by SM-2 beads.

3.3.1. Assays using sqGPAT

In one type of assay we then added G3P and sqGPAT from outside, hoping that we would be able to measure the flipping of acyl-CoA from the inner to the outer leaflet of the liposomal membrane once all [3H]palmitoyl-CoA in the outer leaflet had been consumed. We initially assumed that [3H]palmitoyl-CoA would distribute equally between the outer and inner membrane leaflets during formation of the liposomes and thus expected that 50% acyl-CoA would rapidly be transformed into LPA by externally added sqGPAT. Yet, as shown in Fig. 2, only between 2 and 8% of liposomal [3H]palmitoyl-CoA was transformed into LPA within 1 h. This can be rationalized by assuming that at later stages of liposome formation, SM-2 beads remove [3H]palmitoyl-CoA preferentially from the outer leaflet of liposomes. Experimental evidence for this will be shown below (Fig. 4). As shown in Fig. 2, adding sqGPAT to liposomes from outside we tested spontaneous flipping of acyl-CoA in liposomes made from various lipid mixtures: dioleoyl-PC (DOPC), POPC, POPC/POPE (70:30) and POPC/POPE/DOPS/PI (56:30:7:7), of which the latter more closely resembles the lipid composition of yeast ER membranes. Transbileayer movement of palmitoyl-CoA was generally slow in all these liposomes, with the possible exception of POPC/POPE vesicles that showed slightly more LPA formation in the absence of detergent (Fig. 2). Interestingly, increased LPA formation was already noted 1 min after addition of sqGPAT, with only a moderate further increase during the following 60 min of incubation. It would appear logical to assume that in vesicles allowing slow flipping of acyl-CoA, this flipping may have begun earlier, i.e. at the end of detergent removal and may have continued throughout protease treatment and the assay. Thus, if slow flipping occurs we may expect the kinetics of LPA appearance found for POPC/POPE liposomes in Fig. 2. PE has a propensity to form hexagonal structures in lipid bilayers [31] and may slightly increase the flip-flop of [3H]palmitoyl-CoA (Fig. 2), but the flip-flop rate nevertheless remains very slow, and the phenomenon was only observed with POPC/POPE (70:30), not with POPC/POPE/DOPS/PI (56:30:7:7) liposomes, which also contain PE.

In Fig. 2 we could not use perfringolysin O, as this toxin only perrates Cho-containing bilayers. We therefore used TX-100 as a positive control.

3.3.2. Assays using alkaline phosphatase

As a further protease-free assay we treated the same type of [3H]palmitoyl-CoA-containing liposomes as used in Fig. 2 with phosphatase. Treatment with bovine intestinal alkaline phosphatase (AP) rapidly transforms [3H]palmitoyl-CoA into a slightly more hydrophobic species migrating off the origin in our TLC system (band b in Fig. 3B), whereas prolonged incubation with high doses of AP leads to the appearance of an even more hydrophobic band (band e). We tentatively attribute the first shift to the removal of the 3′-phosphate on the
Adenosine moiety and the second shift to the removal of 3′-phosphoryl-ADP or ADP, although band e was not characterized and may also represent a free fatty acid generated by a contaminating thioesterase (Fig. 3A). As shown in Fig. S4A, lanes 1–5, λ phosphatase did not generate the same dephospho-acyl-CoA (band b) as AP, whether or not BSA was present; therefore it was not utilized further. Slightly varying the pH or temperature had very little effect on the generation of dephospho-[3H]palmitoyl-CoA by AP, but raising the temperature and pH enhanced the generation of band 3 (Fig. S4A, lanes 6–13, Figs. S4B and S4C). The kinetics of the reaction also suggests that most [3H]palmitoyl-CoA is first transformed into dephospho-[3H]palmitoyl-CoA, which then is converted by sqGPAT and AP to [3H]palmitoyl-CoA-containing liposomes resulting in very similar results: As shown in Fig. 3C, using sqGPAT and AP in parallel on the same liposomes at the same molar ratio, we observed that similar amounts (2–7%) of [3H]palmitoyl-CoA were cleaved within 1 h of incubation. Also, the same results were obtained with POPC and POPC/POPE/DOPS/PI liposomes (Fig. 3C). A further indication that sqGPAT and AP produce similar results is documented in Fig. S5, where the liposomes used for sqGPAT assays in Fig. 2 were treated in parallel also with AP. The assays of Fig. S5 again suggested that only POPC/POPE (70:30 mol%) liposomes may allow slow spontaneous flip-flop of acyl-CoA. Further assays also showed that when [3H]palmitoyl-CoA was incorporated into DOPC/Cho or DOPC/Ergosterol liposomes most of acyl-CoA remained in the inner leaflet and exhibited only very slow spontaneous flipping (Fig. S5). In view of the very low amounts of dephospho-[3H]palmitoyl-CoA generated by AP in most liposome assays, we also wanted to make sure that AP could access [3H]palmitoyl-CoA in lipid bilayers in the absence of detergent. As shown in Fig. 4, AP cleaved [3H]palmitoyl-CoA in LUVs having been exposed to PFO quite efficiently (lanes 4, 5 vs. 2, 3), albeit somewhat less efficiently than that in the presence of OGP (lanes 6, 7). TX–100 in many assays was less efficient than OGP in making [3H]palmitoyl-CoA accessible for AP, for unknown reasons (lane 10 vs. 7); this also can be seen in Fig. S5. Further controls showed that detergents or PFO in the absence of AP had no influence on the TLC mobility of [3H]palmitoyl-CoA (Fig. 4, lanes 8, 9 and 11).

3.4. Liposomes having higher amounts of [3H]palmitoyl-CoA in the outer leaflet can be obtained by reducing the second round of detergent removal

As mentioned, we speculated that the low percentage of radioactivity of liposomes containing [3H]palmitoyl-CoA that could be metabolized by enzymes added from outside (Figs. 2, 3C, S5, S6) may reflect preferential removal of [3H]palmitoyl-CoA from the outer leaflet during later stages of liposome formation. To test this idea, we compared the liposomes made as usual, to liposomes made by omitting the second round of detergent removal. As can be seen in Fig. 5, omission of the second incubation with SM-2 beads resulted in a higher percentage of immediately accessible palmitoyl-CoA (lanes 1–10 vs. 11–17). This was very clearly seen using sqGPAT (lanes 7–10 vs. 15–17), since in this case, there was little increase of LPA between 10 and 60 min of incubation. This would indicate a) that already after the first round of detergent removal, the microsomes are relatively stable, and [3H]palmitoyl-CoA flipping already is very slow, in spite of residual detergent in the membranes; and b) that the second detergent removal strongly increases the asymmetric distribution of [3H]palmitoyl-CoA between the inner and outer leaflets, i.e. that it removes [3H]palmitoyl-CoA preferentially from the outer leaflet. Incidentally, the data also argue that AP is reacting much less rapidly with [3H]palmitoyl-CoA than sqGPAT (Fig. 5, lanes 1–5 vs. 6–10). The possibility of shortening the detergent extraction seems to be attractive since it yields a higher recovery of [3H]palmitoyl-CoA in liposomes.

4. Discussion

We believe that the assays described in this report will allow incorporating purified integral membrane proteins into LUVs by adding a
Fig. 3. Phosphatase treatment of large unilamellar vesicles containing $[^{3}H]$palmitoyl-CoA. A, structure of palmitoyl-CoA. B, $[^{3}H]$palmitoyl-CoA (17 pmol) was treated with 10 or 100 U of AP for indicated times (min) at RT and pH 7.0; products were analyzed by TLC and radioscanning. 1.7 fold increments in density of radioactivity are differentiated by different colors. In lane 9, AP had been boiled. C, liposomes (100 nmol of POPC or POPC/POPE/DOPS/PI) containing 17 nmol $[^{3}H]$palmitoyl-CoA were incubated with 10 U of AP in the same conditions as in panel B, including either OGP (○) or TX-100 (T) as indicated. Amounts of products (b = LPA or dephospho-$[^{3}H]$palmitoyl-CoA and e) were calculated as percentage over the total radioactivity in the lane.
detergent solubilized protein to the starting mixture. In here, all LUVs were made from lipid/OGP mixtures but LUVs from lipid detergent mixtures containing C12E9 or TX-100 gave very similar results, and these latter detergents may be advantageous for certain proteins. It seems worth noting that also most LUVs lacking cholesterol, a lipid believed to stabilize lipid bilayers, did not allow flipping of palmitoyl-CoA.

The various liposomal assays described here are complementary, since encapsulating GPAT can measure the transfer of acyl-CoA from outside to the inside, whereas the assay using empty liposomes having \[^3H\]palmitoyl-CoA concentrated in the inner leaflet allows measuring the transfer in the opposite direction. The former assay however is less universal, as it requires protease to remove the sqGPAT sticking to the outer leaflet. This step really is crucial as it removes substantial amounts of sqGPAT (Fig. S1, lane 2 vs. 3). Yet, we found that adding Ni-NTA beads to LUVs can eliminate 90% of passively absorbed sqGPAT (not shown). Our assays use radiolabeled acyl-CoA in order to keep the chemical amounts of acyl-CoA low, but as shown in Fig. S3, acyl-CoA does not seem to greatly perturb the bilayer structure and it therefore

![Fig. 4. Phosphatase treatment of large unilamellar vesicles (DOPC/Cho) containing \[^3H\]palmitoyl-CoA in presence of PFO or detergent. Liposomes (DOPC/Cho, 70:30 mol%) containing \[^3H\]palmitoyl-CoA (17 pmol) were preincubated with PFO, OGP or TX-100 for 10 min and further treated with 10 U of AP for indicated times (min) at RT and pH 7. Products were analyzed by TLC and radioscanning. The amount of dephospho-palmitoyl-CoA formed is indicated as % dephospho-palmitoyl-CoA/total radioactivity in the lane (b/total). PFO incubated with LUVs did not result in any degradation of \[^3H\]palmitoyl-CoA (lane 11). Lanes 1–10 are from a single experiment and a single TLC, but rearranged to give a more logical order. Red lines separate lanes that are not adjacent on the original TLC.](http://doc.rero.ch)

![Fig. 5. Incomplete detergent removal results in a higher amount of \[^3H\]palmitoyl-CoA in the outer leaflet of liposomes. A mixture of 50 pmol OGP, 5 pmol lipids (POPC) and 530 pmol \[^3H\]palmitoyl-CoA was first incubated with 150 mg SM-2 beads for 2 h at RT on a rotating wheel at 4 °C, an additional 300 mg beads were added and incubation continued for 2 more hours. Beads were removed and half the sample was set aside for assays shown in lanes 1–10 (1 × SM-2 beads); the other half was further incubated with fresh 250 mg beads for 2 h at 4 °C (2 × SM-2 beads). Immediately thereafter, both liposome preparations were used for the assays. Assays contained either 30 U AP (lanes 2–5, 12–14) or 8.8 μg of sqGPAT/100 nmol G3P (lanes 7–10, 15–17) and were incubated for the indicated times (min). The amounts of liposomes (incubated 1 × or 2 × with SM-2 beads) were adjusted such that all samples contained 17 pmol (1 μCi) \[^3H\]palmitoyl-CoA. Products were analyzed as in Fig. 3 and the amount of product calculated as in Fig. 1.](http://doc.rero.ch)
may be feasible to use higher amounts of acyl-CoA. This could open the possibility of using bioorthogonal chemistry to follow the enzymatic reactions.

Conceptually it has to be considered that certain acyltransferases would flop acyl-CoA themselves. We expect that the assays described in here are very suitable to demonstrate such an activity as long as the flipase activity does not depend on the acyltransferase activity through a stringent substrate channeling mechanism and as long as the substrates of the tested acyltransferase is not present in the liposomes.

We hope this assay will be suitable to understand how MBOAT proteins and other acyltransferases of the secretory pathway get their substrate.

**Transparency document**

The Transparency document associated with this article can be found, in the online version.

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**Appendix A. Supplementary materials**

Supplementary data to this article can be found at http://dx.doi.org/10.1016/j.jbbamem.2015.08.020.

**References**


