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Supplementary materials

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

Andrej Bavdek, Hector M. Vazquez* and Andreas Conzelmann**

Supplemental experimental procedures

Reagents. 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), and palmitoyl-Coenzyme A (ammonium salt) (16:0) were purchased from Avanti Polar Lipids. 1,2-Diacyl-sn-glycero-3-phospho-(1-D-*myo*-inositol) (PI), n-octyl-β-D-glucopyranoside (OGP), TX-100, 5,5'-dithiobis-(2-nitrobenzoic acid) (≥98%) (DTNB, Elman's reagent), sn-Glycerol 3-phosphate bis(cyclohexylammonium) salt (G3P), bovine serum albumin BSA, Acyl-Coenzyme A synthetase from *Pseudomonas sp.* (A3352), and inorganic Pyrophosphatase from baker's yeast (I1891) were acquired from Sigma-Aldrich. Alkaline phosphatase from bovine intestinal mucosa (79385), calcein (21030), Adenosine 5′-triphosphate disodium salt hydrate (ATP), 1-butanol (n-butanol) and hexane were purchased from Fluka. Bio-Beads SM-2 were acquired from Bio-Rad. Ni-NTA Agarose was purchased from QIAGEN. Q Sepharose Fast Flow was acquired from GE Healthcare. TCEP (11303) was purchased from CovaChem, USA. α-dodecyl-ω-hydroxy-poly(oxy-1,2-ethanediyl oxy-1,2- ethanediyl) (C12E9) was acquired from Affymetrix, USA. [^{3}H]palmitic acid [9,10-3H(N)] (10 mCi/ml; 60 Ci/mmol) was purchased from ANAWA Trading SA, Wangen, Switzerland. Coenzyme A trilithium salt (CoA; 234101) and 1,2-dichloroethane were acquired from Merck. Silica gel 20 x 20 cm TLC plates were obtained from Macherey-Nagel, Switzerland. LabAssay Phospholipid (296-63801) to measure PC was from Wako, Japan.

Cloning, expression and purification of sqGPAT. The coding region of the chloroplast GPAT of squash (sqGPAT) was amplified by PCR directed by the oligonucleotides sqGPAT-forward (5'-

GGC AGC CAT ATG GCT AGC CAC TCC CGC AAA TTT CTC -3') and sqGPAT-reverse (5'- AGC AGC CGG ATC CTC GAG CTA CCA AGG TTG TGA CAA AGA G -3') using cDNA from *Cucurbita moschata* (SQ1AT cDNA (AT03) in pTZ18R as the template. The PCR product was cleaved with *Nde*I and *Xho*I, and inserted into a precleaved T7-promoter-based expression vector, pET15b, providing a His_6-tag at the N-terminus. The correct construction of the plasmid was verified by nucleotide sequencing. sqGPAT was overexpressed in the *E. coli* BL21(DE3) strain (Novagen, USA). A 50 mL sample of overnight culture was used to inoculate 2 L of Luria–Bertani medium containing 100 µg/ml of ampicillin. Expression of sqGPAT was induced at an OD_{600} of approximately 0.8 by adding isopropyl thio-β-D-galactoside to a final concentration of 1 mM. Cells were grown for an additional 5 h at 30 °C, centrifuged and frozen at -20 °C. Six g of frozen cells, was thawed into 50 mL buffer A (20 mM potassium phosphate, pH 8, 300 mM NaCl, 10% glycerol) supplemented with 1 mM TCEP, 1 mg/ml of lysozyme, 10 µg/ml of DNase, 20 µg/ml of RNase and 1x Roche protease inhibitor cocktail (EDTA-free). The cells were incubated on ice for 45 min with occasional vigorous shaking, and extracted by passing 5 times through the ice-precooled Microfluidizer M-110L (Microfluidics). Broken cells were centrifuged (30 min, 4 °C, 15 000 g). Supernatant was applied to a 2-mL Ni-NTA gravity column (QIAGEN), which had been equilibrated with buffer A containing 10 mM imidazole. Unbound proteins were eluted with the above buffer and the bound sqGPAT was eluted with buffer A containing 300 mM imidazole. The protein was dialyzed two times against 3 L of buffer B (20 mM potassium phosphate, pH 8, 50 mM NaCl) at 4 °C. The dialyzed sample was applied to a 1-ml ion-exchange Q Sepharose Fast Flow column (GE Healthcare) equilibrated with buffer B. Bound sqGPAT was eluted from the column by a linear gradient of NaCl in buffer B up to 1 M NaCl. Fractions shown to contain sqGPAT by SDS-PAGE (12% gels) and Coomassie blue staining were pooled and stored at -80 °C in buffer B.

Cloning, expression and purification of PFO. The coding region for the Cho binding perfringolysin O (PFO) from *Clostridium perfringens* without the signal sequence was amplified by PCR using the oligonucleotides PFO-forward (5'-GCGGATCCAAGGATATAACAGATAAAAATCAAAGTAT-3') and PFO-reverse (5'- CGC

ACG CGT TTA ATT GTA AGT AAT ACT AGA TCC AGG-3'). The PCR product was cleaved with *Bam*HI and *Mlu*I, and inserted into a precleaved, T7-promoter-based expression vector that leaves a His_6-tag with a thrombin-recognition sequence at the N-terminus [32]. The correct construction of the plasmid was verified by nucleotide sequencing. PFO was overexpressed in the *E. coli* BL21(DE3) strain and purified using exactly the same protocol as for sqGPAT except that only 0.5 mM thio-β-D-galactoside were used.

Colorimetric assay for sqGPAT activity. Enzymatic activity of sqGPAT was determined by colorimetric measurements at 412 nm [33]. The reaction was carried out at RT in buffer D containing 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Elman's reagent) in a spectrometric cuvette. Assays contained 2 µg (46 pmol) of sqGPAT, 1 mM G3P and 1-20 µM palmitoyl-CoA in a final volume of 1 ml. Note that the critical micelle concentration (CMC) of palmitoyl-CoA under similar conditions is 75 µM [26]. Equimolar concentrations of BSA were added to insure solubility of palmitoyl-CoA. Reagents were added into the cuvette in the order: BSA, liposomes or detergent, sqGPAT, G3P, palmitoyl-CoA (dissolved in water).

Synthesis of [^{3}H]palmitoyl-CoA. [^{3}H]palmitoyl-CoA was synthesized adapting published methods [34, 35] in 1 ml of buffer C (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 0.1 mM EGTA, 1 mM DTT, 10 mM ATP, 10 mM CoA, 1.7 mM (0.1%) Triton X-100) containing 0.1 U Acyl-Coenzyme A synthetase, 1 U Pyrophosphatase and 40 nmol (2.4 mCi) [^{3}H]palmitic acid. For this, [^{3}H]palmitic acid was gently dried under a flow of N_2 in a glass tube. 1 ml of buffer C containing the enzymes was added. After vigorously vortexing for 2 min the tube was left 3 h at room temperature (RT). [^{3}H]palmitoyl-CoA was extracted 5 times with 1 ml water saturated butanol (1:1, v/v) and dried in a rotary evaporator (SpeedVac). The dried material was resuspended in 1 ml of water-saturated 1,2-dichloroethane by vortexing; 1 ml of 1,2-dichloroethane-saturated water was added and the sample again vortexed vigourosly. Dichloroethane extracts free [^{3}H]palmitic acid and TX-100, whereas [^{3}H]palmitoyl-CoA remains in the water phase. The sample was then centrifuged at 5000*g* for 5 min. The (lower) dichloroethane phase was carefully discarded by a Pasteur pipette. The water phase containing [^{3}H]palmitoyl-CoA was extracted four more times with water-saturated dichloroethane. Finally,

[^{3}H]palmitoyl-CoA was removed from the water phase by adding 1 ml water saturated 1-butanol and the butanol phase was dried under the vacuum. The dried material was finally resuspended in 4 ml of chloroform-methanol-water 80:20:2 or water-saturated butanol by intensive vortexing and stored at -20°C. The typical yield of [^{3}H]palmitoyl-CoA was around 50 % in terms of the [^{3}H]palmitate added in the beginning.

Colorimetric assay for sqGPAT activity. Enzymatic activity of sqGPAT was determined utilizing a spectrophotometer Cecile CE1010 (Cecile Instruments) and measuring the absorption at 412 nm. Reaction were carried out in buffer 1 ml of D containing 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The sqGPAT concentration in the cuvette was 2 μg/ml. Normally, concentrations of substrates were 1 mM G3P and 1-20 μM palmitoyl-CoA. Equimolar concentrations of BSA were added to insure palmitoyl-CoA solubility and accessibility. Reagents were added into the cuvette in the order: Buffer, BSA, liposomes or detergent, sqGPAT, G3P, palmitoyl-CoA, respectively. Reactions were allowed to take place at RT.

Calcein release. Large unilamellar vesicles (LUVs) containing calcein were prepared by freeze thawing and extrusion of multilamellar vesicles as described [27]. Briefly, 2.1 μmol of DOPC and 0.9 μmol of Cho (70:30 mol%) in chloroform were mixed and dried in a 30 ml Pyrex round-bottom glass tube in a rotary evaporator under vacuum for 3 h. The lipid film was resuspended in 1 ml of 60 mM calcein in buffer D and vigorously vortexed in the presence of glass beads for 2 min, yielding multilamellar vesicles (MLV). MLVs were then frozen and thawed repeatedly (six times) by shifting from liquid N_2 to a water bath at 37°C. Then, MLVs were extruded through polycarbonate membranes with 100 nm pores by an Avestin lipid extruder (Avestin, Ottawa, ON) to yield LUVs with 100 nm in diameter [36]. The excess of calcein was removed by gel filtration on a small G-50 column. The concentration of PC was determined by an enzymatic test based on the choline oxidase/DAOS method [24]. Permeabilization of calcein-loaded LUVs was assessed by using a fluorescence reader. For this, LUVs representing 5 nmol of DOPC/Cho (70:30 mol%) were added into 1 ml of buffer D and placed in a fluorimetric cuvette under constant magnetic stirring. Repeated additions of palmitoyl-CoA were made. The excitation and emission filters were set to 485 and 535 nm, respectively. The permeabilization

induced by palmitoyl-CoA was compared to the maximal permeabilization obtained at the end of the assay by the addition of detergent TX-100 to a final concentration of 2 mM.

Figure S1

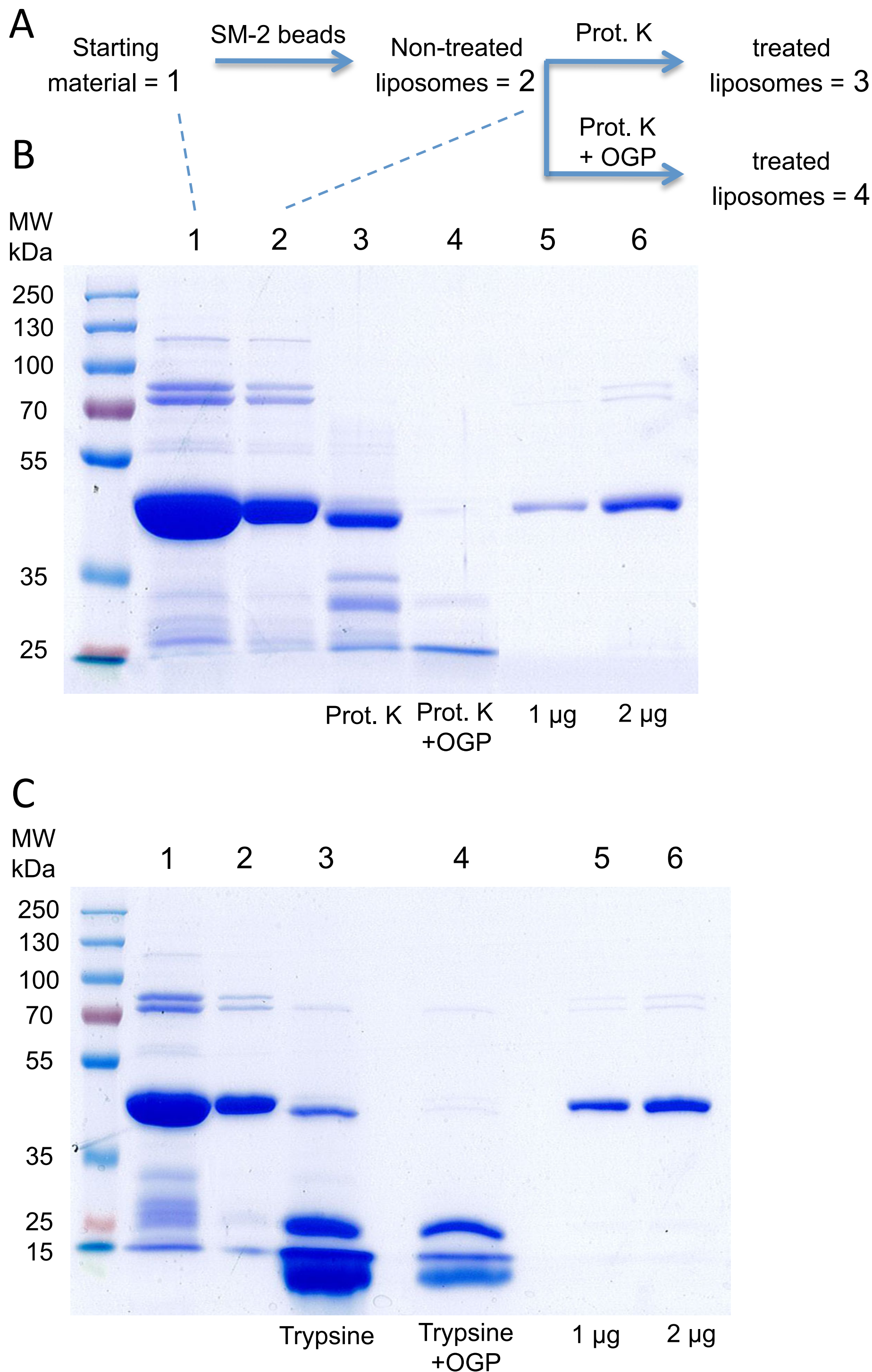
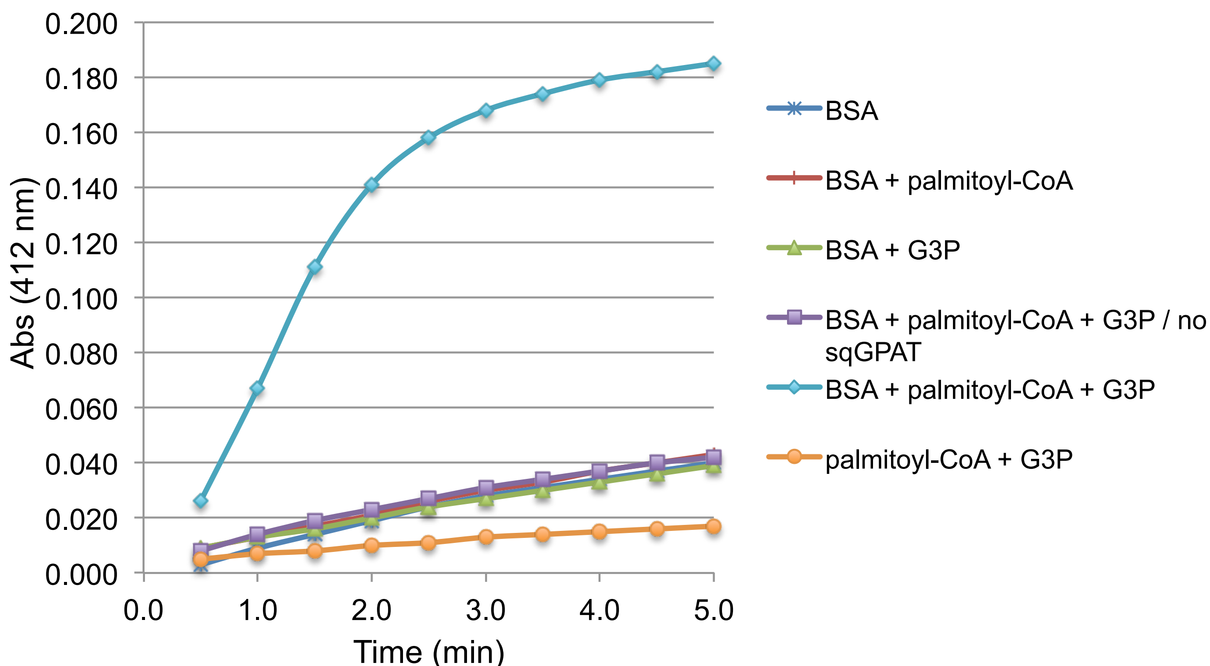


Figure S1. Sequential steps of sqGPAT encapsulation into LUV. A, different stages, at which samples were taken for SDS-PAGE. Prot. K, proteinase K.
B, SDS-PAGE and Coomassie staining of samples taken at different stages during liposomes preparation. Lane 1, starting material before removal of detergent; lane 2, liposomes after complete removal of detergent with SM-2 beads; lane 3, liposomes after proteinase K treatment for 1 h at 4°C; lane 4, liposomes after proteinase K treatment for 1 h at 4°C in presence of OGP; lanes 5 and 6, 1 µg and 2 µg of sqGPAT for comparison. C, lanes as in panel A, but liposomes were treated with 50 µg of trypsine.

Figure S2

A



B

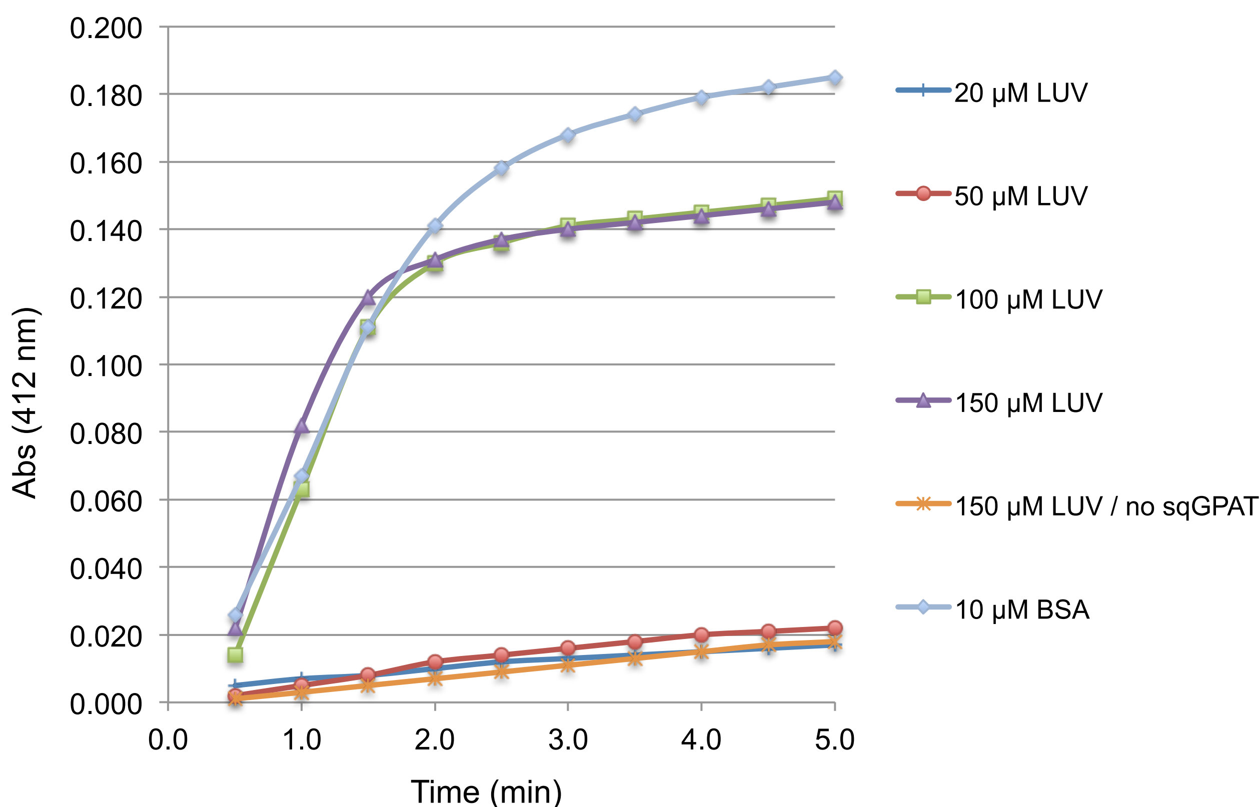


Figure S2. Effect of the addition of BSA or liposomes on sqGPAT enzymatic activity in solution. A and B, reactions were carried out in 1 ml of buffer D with 1 mM DTNB. Reagents were added in the order: 10 µM BSA (panel A) or LUVs (panel B) equivalent to 20 - 150 µM of DOPC, 2 µg sqGPAT, G3P (to 1 mM), and palmitoyl-CoA (to 10 µM). For this experiment, LUVs were made by extrusion (without using detergent). A 10 µM solution of CoA also gave an absorbance of about 0.2, indicating that the reaction with BSA goes to completion. The experiment was repeated once and gave the same result.

Figure S3

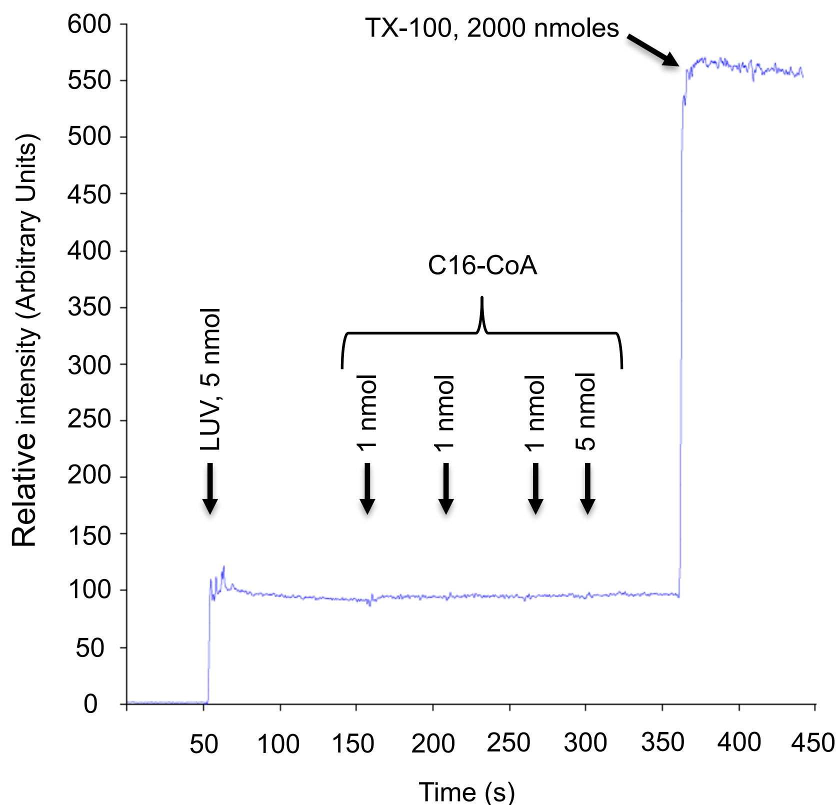


Figure S3. *Permeabilisation of calcein-loaded LUVs by palmitoyl-CoA*. Calcein containing liposomes equivalent to 5 nmol of DOPC in 1 ml of buffer D were placed in a photometric cuvette and kept under constant stirring at 25°C. At various times, the indicated amounts of palmitoyl-CoA and TX-100 were added from 20 µM and 20% (v/v) stock solutions. Fluorescence was followed by using an excitation wavelength of 485 nm and measuring the emission at 520 nm. TX-100 does not absorb/emit or significantly disperse at these wave lengths and concentration.

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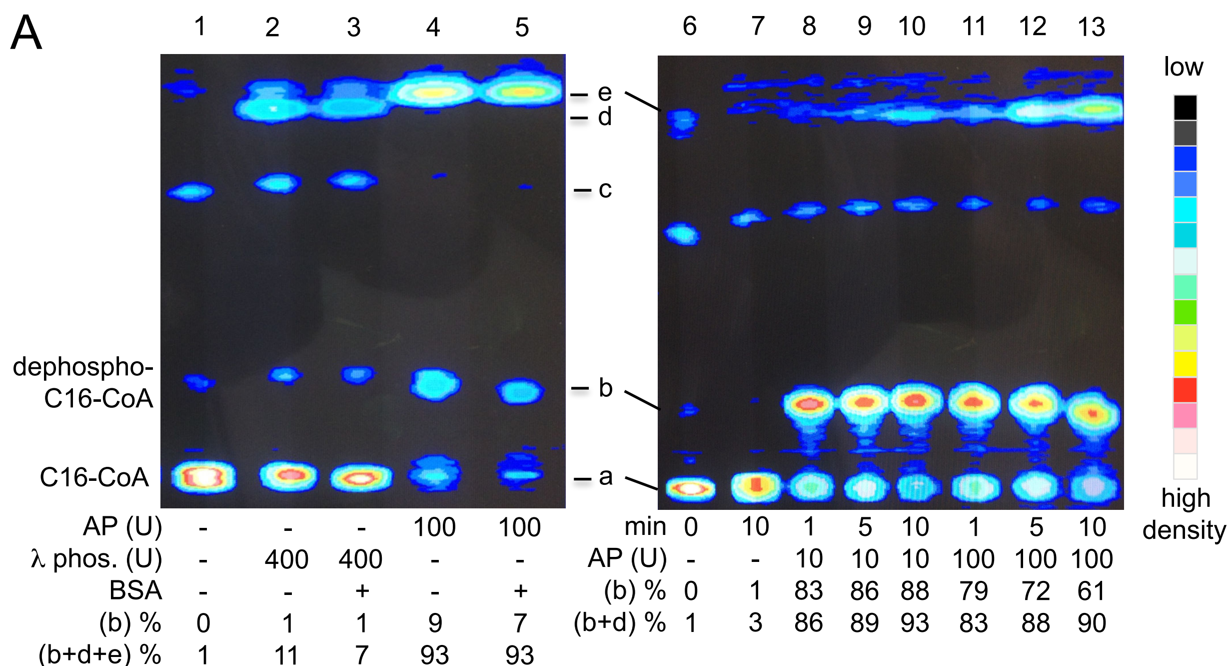
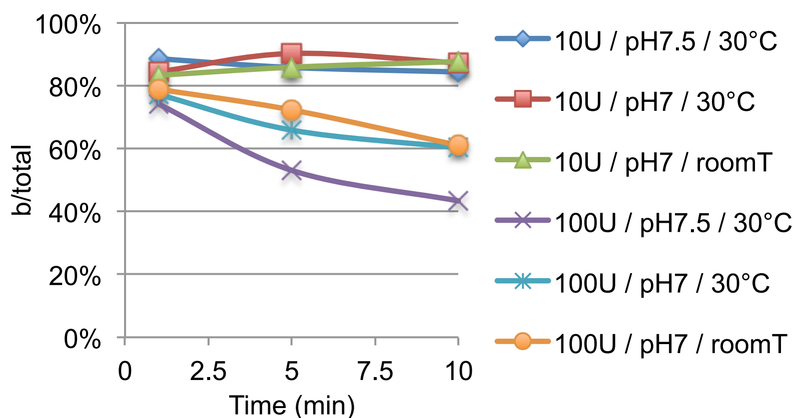
Figure S4





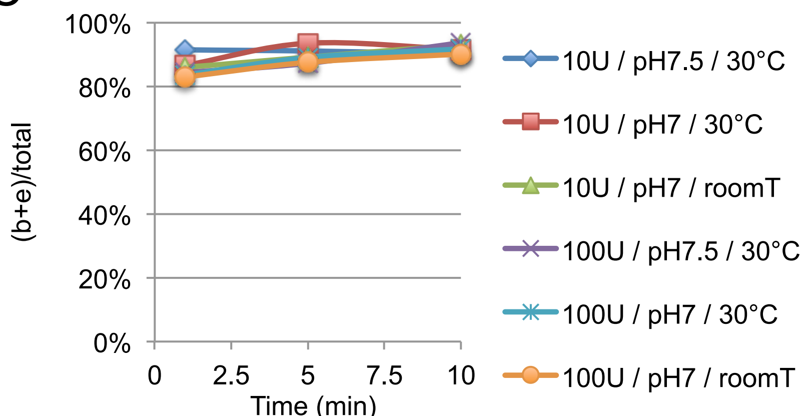


Figure S4. *Phosphatase treatment of [^{3}H]palmitoyl-CoA in the absence of liposomes.* A, lanes 1 - 5: [^{3}H]palmitoyl-CoA (17 pmol) was treated with bovine alkaline phosphatase (AP, 100 U) or λ phosphatase (400 U) at 30°C for 1 h at pH 7.5. Lanes 6 – 13, [^{3}H]palmitoyl-CoA was treated with 10 or 100 U AP for 0 to 10 min at RT and pH 7.0. Products were analyzed by TLC and radioscanning as in Fig. 3. B, and C: Formation of dephospho-[^{3}H]palmitoyl-CoA by phosphatase treatment in 20 mM phosphate buffer with various concentrations of AP, at various pH (pH 7 or 7.5) and temperatures (RT or 30°C). Panels B and C plot the data of Fig. 3B, S4A (lanes 6 – 13) and an additional experiment.

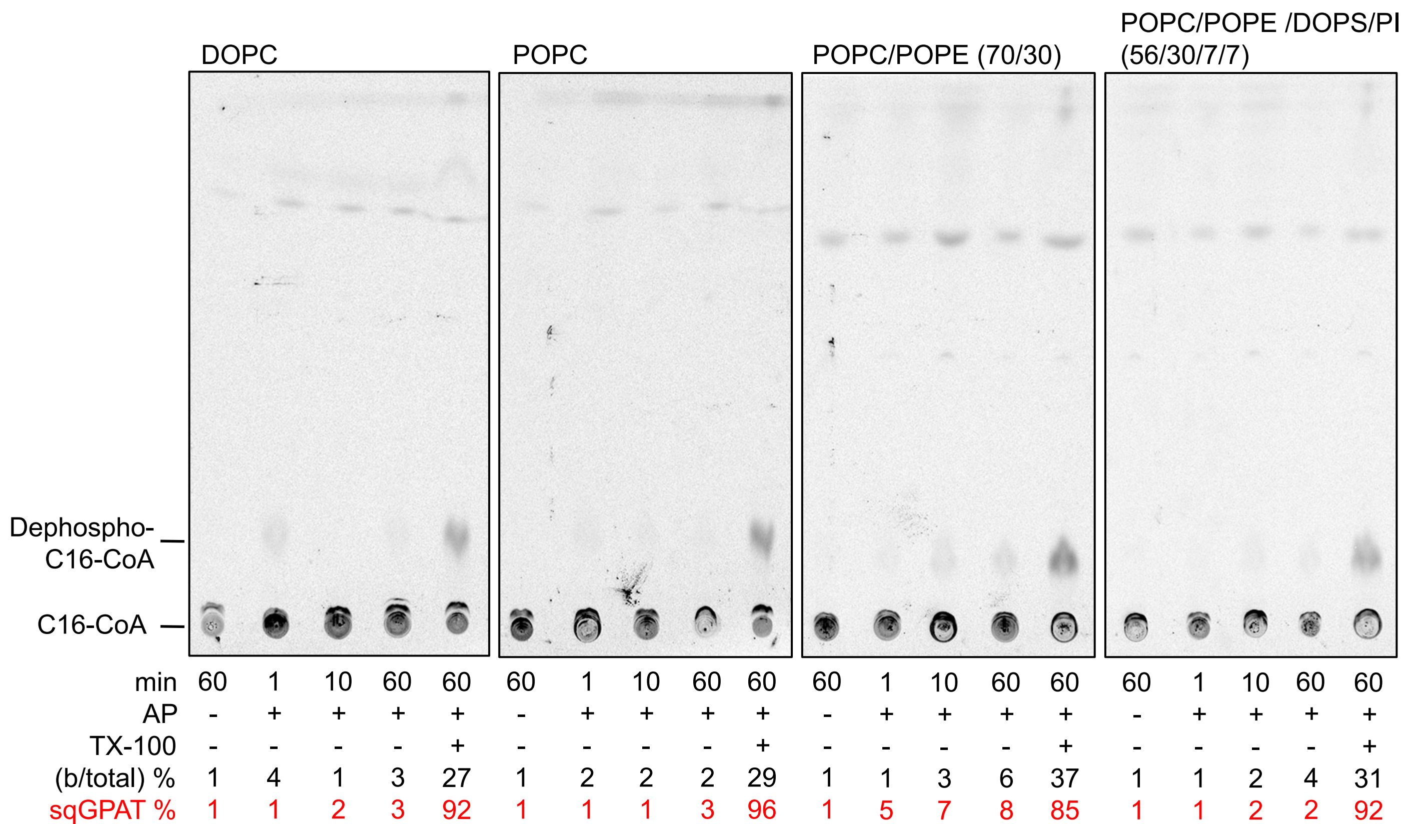


Figure S5. *Phosphatase treatment of large unilamellar vesicles of different composition containing [^{3}H]palmitoyl-CoA.* The same LUVs as used for Fig. 2 (100 nmol of DOPC, POPC, POPC/POPE, POPC/POPE /DOPS/PI) containing [^{3}H]palmitoyl-CoA (17 pmol) were diluted into 1 ml of buffer D (pH 7.0) containing 10 U of AP and incubated at RT for indicated times (min). The fraction of dephospho-palmitoyl-CoA was determined by TLC/radioscanning and calculated as percentage of total radioactivity per lane.
Note that the use of TX-100 is not optimal for AP, always resulting in lower values for maximal cleavage than OGP (Fig. 3C). For comparison, the % of LPA generated by sqGPAT using the same liposomes (Fig. 2) are added in red.

Figure S6

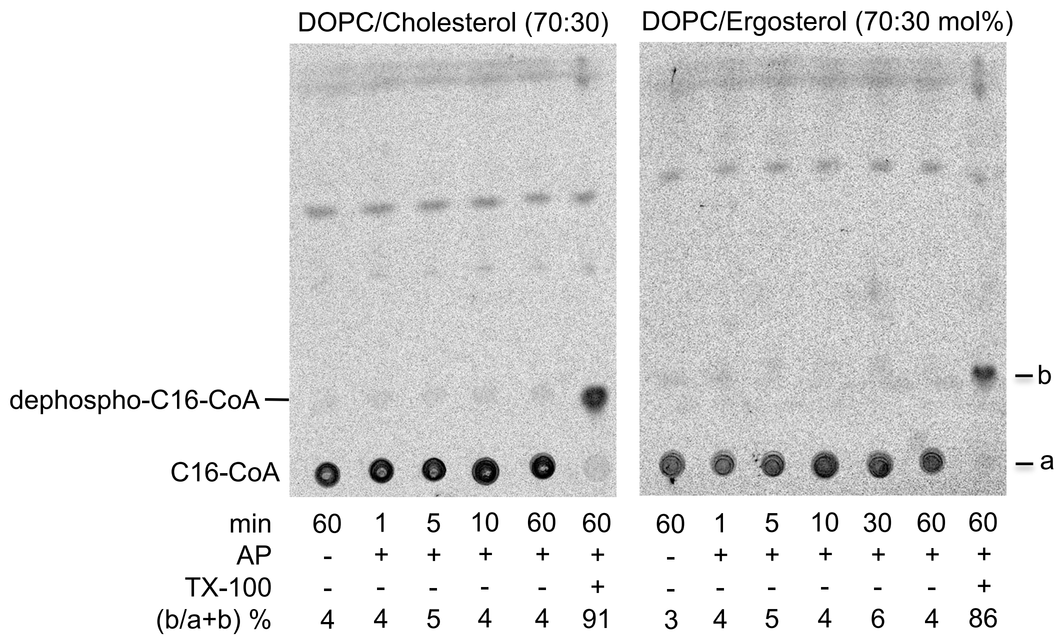


Figure S6. *Phosphatase treatment of large unilamellar vesicles containing cholesterol or ergosterol and [^{3}H]palmitoyl-CoA.* LUVs (100 nmol of DOPC/Cholesterol (70:30 mol%), or DOPC/Ergosterol (70:30 mol%)) containing [^{3}H]palmitoyl-CoA (17 pmol) were treated with 10 U of alkaline phosphatase at pH 7.0 and RT. Reactions were incubated for indicated times (min). The amount of dephospho-Palmitoyl-CoA formed is indicated as % dephospho-palmitoyl-CoA/[palmitoly-CoA + dephospho-palmitoyl-CoA].