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Transbilayer Movement of Dipalmitoylphosphatidylcholine in Proteoliposomes Reconstituted from Detergent Extracts of Endoplasmic Reticulum

KINETICS OF TRANSBILAYER TRANSPORT MEDIATED BY A SINGLE FLIPPASE AND IDENTIFICATION OF PROTEIN FRACTIONS ENRICHED IN FLIPPASE ACTIVITY*

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Phospholipid translocation (flip-flop) across membrane bilayers is typically assessed via assays utilizing partially water-soluble phospholipid analogs as transport reporters. These assays have been used in previous work to show that phospholipid translocation in biogenic (self-synthesizing) membranes such as the endoplasmic reticulum is facilitated by specific membrane proteins (flippases). To extend these studies to natural phospholipids while providing a framework to guide the purification of a flippase, we now describe an assay to measure the transbilayer translocation of dipalmitoylphosphatidylcholine, a membrane-embedded phospholipid, in proteoliposomes generated from detergent-solubilized rat liver endoplasmic reticulum. Translocation was assayed using phospholipase A_2 under conditions where the vesicles were determined to be intact. Phospholipase A_2 rapidly hydrolyzed phospholipids in the outer leaflet of liposomes and proteoliposomes with a half-time of ~0.1 min. However, for flippase-containing proteoliposomes, the initial rapid hydrolysis phase was followed by a slower phase reflecting flippase-mediated translocation of phospholipids from the inner to the outer leaflet. The amplitude of the slow phase was decreased in trypsin-treated proteoliposomes. The kinetic characteristics of the slow phase were used to assess the rate of transbilayer equilibration of phospholipids. For 250-nm diameter vesicles containing a single flippase, the half-time was 3.3 min. Proportionate reductions in equilibration half-time were observed for preparations with a higher average number of flippases/vesicle. Preliminary purification steps indicated that flippase activity could be enriched ~15-fold by sequential adsorption of the detergent extract onto anion and cation exchange resins.

The topology of phospholipid biosynthesis in biogenic (self-synthesizing) membranes, such as the eukaryotic endoplasmic reticulum (ER),[1] is such that newly synthesized phospholipids are initially located in the cytoplasmic leaflet of the membrane bilayer (1). To populate the exoplasmic leaflet of the bilayer and allow for uniform bilayer growth, some of the newly synthesized phospholipids have to be transferred (flipped) to the opposite membrane leaflet. Flip-flop does not occur spontaneously because of the thermodynamic barrier encountered in moving the charged/polar headgroup of a phospholipid through the hydrophobic interior of the bilayer. Indeed, in artificial bilayers, liposomal systems, and certain biomembranes, transverse movement of phospholipids, *i.e.* transfer of a phospholipid from one side of the membrane to the other, occurs only very slowly if at all (2–4). Nevertheless, it is clear from a number of studies, including our own, that transbilayer movement of phospholipids occurs rapidly in the ER as well as in other biogenic membranes such as the bacterial cytoplasmic membrane (5–12). These studies further indicate that phospholipid translocation in the ER occurs bidirectionally by a facilitated diffusion process requiring no metabolic energy input. The latter observation rules out the participation of the ABC family of transporters that are involved in metabolic energy-dependent, vectorial transport of solutes and some lipids. Thus the molecular mechanism by which phospholipids are translocated across the ER is unknown.

It is commonly hypothesized that the metabolic energy-*in*dependent flipping of phospholipids across the ER bilayer is facilitated by specific transport proteins (termed biogenic membrane flippases) (13–16). A recent publication from our laboratory (17) described a reconstitution system in which a detergent extract of ER membranes was used to prepare proteoliposomes capable of transporting dibutyroyl-PC, a water-soluble analog of phosphatidylcholine. This reconstitution system was used to show that dibutyroyl-PC translocation required a specific membrane protein(s) whose abundance was estimated at ~0.2% by weight of ER membrane proteins. We now extend these results to assay the transbilayer movement of a genuinely membrane-embedded phospholipid, dipalmitoylphosphatidylcholine (DPPC). The new assay, based on phospholipase A_2 (PLA_2)-mediated hydrolysis of glycerophospholipids located in the outer leaflet of membrane vesicles, confirms the main conclusions of our previous work while eliminating the real and perceived complications of using short chain phospholipid analogs as transport reporters.[2] The assay also offers the opportunity to measure the kinetics of transport, a measurement that was not possible with the dibutyroyl-PC-

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[1] The abbreviations used are: ER, endoplasmic reticulum; DPPC, dipalmitoyl-PC; ePC, egg phosphatidylcholine; FITC, fluorescein isothiocyanate; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2; PS, phosphatidylserine; SWER, salt-washed ER; TE, Triton X-100 extract.

[2] The initial comparison of the dibutyryl-PC-based assay with the PLA_2-based assay was described by Hrafnsdóttir and Menon (18).

based assay. Thus we report on the requirement for a specific, trypsin-sensitive ER protein(s) to translocate DPPC as well as on the rate at which DPPC equilibrates between the inner and outer leaflets of vesicles equipped with a single translocator/flippase. We conclude by using our assay procedures to show that flippase activity can be readily enriched by biochemical fractionation of the ER detergent extract, thus laying the groundwork for eventual purification of the transporter.

EXPERIMENTAL PROCEDURES

Materials—L-α-Dipalmitoyl-[^{3}H]phosphatidylcholine (~80 Ci/mmol) and D-[2-^{3}H]mannose (~20 Ci/mmol) were from American Radiolabeled Chemicals, Inc. Frozen rat livers were purchased from Pel-Freez Biologicals. SM-2 Bio-Beads for detergent removal and all chemicals required for SDS-PAGE were purchased from Bio-Rad Laboratories. ULTROL™ grade Triton X-100 and CM-Sepharose fast flow resin were purchased from Calbiochem. Egg phosphatidylcholine (ePC), phosphatidylethanolamine (PE), phosphatidylserine (PS), PLA_2 from *Naja naja* venom, FITC-dextran (molecular mass ~ 4,300 Da) and routine chemicals were obtained from Sigma Chemical Co. unless otherwise specified. Silica 60 thin layer chromatography (TLC) plates were from EM Science. All chemicals were analytical grade unless otherwise specified.

Preparation of Salt-washed ER (SWER) Microsomes—Rough ER microsomes were isolated from rat liver homogenates and stripped of peripheral proteins by salt washing as described previously (17). Commercially available frozen rat livers were used for this purpose. The SWER preparations were resuspended in 0.25 M sucrose, 50 mM HEPES-NaOH, pH 7.5, at a protein concentration of ~20–25 mg/ml. Aliquots (0.5 ml) were stored frozen at −80 °C and thawed rapidly in a 30 °C water bath before use. The yield of SWER was ~120 mg/g, wet weight, of liver.

Preparation of a Triton X-100 Extract (TE) of SWER Membranes—TEs were prepared freshly for every experiment by mixing equal volumes of SWER and solubilizing buffer (50 mM HEPES-NaOH, pH 7.5, 200 mM NaCl, 2% (w/v) Triton X-100). The mixture was incubated for 30–60 min on ice with occasional pipette mixing before being centrifuged in a TLA 100.2 rotor at 70,000 rpm (175,000 × *g*) for 30 min. The clear supernatant (TE) was removed carefully using a Pasteur pipette without disturbing the fluffy surface of the pellet of Triton-insoluble material. The TE was placed on ice until use for chromatography or vesicle reconstitution. The TE typically contained ~65% SWER protein.

Reconstitution Protocol—Proteoliposomes were prepared from a mixture of TE (or TE-derived chromatographic fractions) and Triton X-100-solubilized ePC. Protein-free liposomes were prepared similarly from Triton X-100-solubilized ePC alone or ePC supplemented with other phospholipids or a lipid extract of SWER. The mixtures were supplemented with [^{3}H]DPPC to a concentration of ~1.25 μCi of [^{3}H]DPPC/μmol of ePC. Briefly, ePC and [^{3}H]DPPC were dried under nitrogen in a glass screw cap tube, and the dried residue was dissolved in reconstitution buffer (10 mM HEPES-NaOH, pH 7.5, 100 mM NaCl, 1% (w/v) Triton X-100). TE or TE-derived fractions were added as specified to yield 2 ml of sample in reconstitution buffer at a phospholipid concentration of 4.5 mM. Different amounts of TE were used to generate proteoliposomes with different protein/phospholipid ratios. The samples were then treated with SM-2 Bio-Beads as described previously (17–19) to remove the Triton X-100 and generate liposomes or proteoliposomes. The vesicles were collected by centrifugation in a TLA 100.3 rotor for 45 min at 80,000 (~230,000 × *g*) rpm and 4 °C, resuspended in HS buffer (10 mM HEPES-NaOH, pH 7.5, 100 mM NaCl), and centrifuged again as described above. The washed vesicles were suspended in HS buffer, and transport assays were carried out the same day. Protein recovery in the reconstituted vesicles was ~50%; lipid recovery was ~70%.

Transport Assay—Transbilayer translocation of [^{3}H]DPPC in reconstituted vesicle preparations was measured by determining the accessibility of the lipid to exogenously added PLA_2 (for a schematic illustration of the assay, see Fig. 1). Suspensions of [^{3}H]DPPC-containing reconstituted vesicles were adjusted to a concentration of 0.1 mM phospholipid, supplemented with $CaCl_2$ (5 mM final) and NaCl (180 mM final), then incubated with PLA_2 from *N. naja* venom (~1 unit/μl) at 30 °C for different periods of time. The total assay volume was 30 μl. Hydrolysis was stopped by adding EGTA (30 μl from a 120 mM stock solution) and 40 μl of water. Phospholipids were immediately extracted according to the procedure of Bligh and Dyer (20) and analyzed by TLC on silica 60 plates using chloroform, methanol, and 28% ammonia (65:25:5, by volume) as the solvent system. Radioactivity on the TLC plate was detected and quantitated with a Berthold LB 2842 TLC scanner. Samples in which buffer was used instead of PLA_2, or EGTA and buffer were added before the addition of PLA_2, were used as controls. In addition, vesicles were disrupted by adding 0.5% Triton X-100 before the addition of PLA_2 to determine maximum hydrolysis. The extent of hydrolysis was determined as (lyso-[^{3}H]PC)/(lyso-[^{3}H]PC + [^{3}H]DPPC).

Protease Treatment—[^{3}H]DPPC-containing proteoliposomes were prepared from a mixture of ePC, [^{3}H]DPPC, and TE such that the protein/phospholipid ratio was <60 mg/mmol. The reconstituted vesicles (suspended in HS at a concentration of ~1 mM phospholipid) were treated with trypsin (0.3–0.9 mg/ml final concentration as specified) at room temperature for 30 min. The reaction was stopped by adding ~0.6–1.8 mg/ml trypsin inhibitor (in proportion to the amount of trypsin used). Parallel samples were mock-treated with HS buffer instead of trypsin or by adding trypsin inhibitor together with trypsin. Trypsin-treated and mock-treated samples were assayed as described above for their ability to translocate [^{3}H]DPPC.

Vesicle Integrity—Vesicle integrity was monitored by determining the extent of leakage of soluble reporters trapped within the vesicles. The leakage of radiolabeled reporters such as [^{3}H]mannose and [^{3}H]inulin was determined as described previously (17, 18). Additional tests were conducted using the fluorescent molecule FITC-dextran (molecular mass ~ 4,300 Da) trapped at self-quenching concentrations within the vesicles. Leakage of FITC-dextran results in dequenching and a consequent increase in fluorescence. Vesicles were reconstituted in the presence of 0.1 mM FITC-dextran and washed by centrifugation to remove nonenclosed FITC-dextran. FITC-dextran-containing vesicles were treated with PLA_2 to measure [^{3}H]DPPC transport. Mock-treated samples were used as controls. After PLA_2 treatment for different periods of time, the vesicles were diluted with HS buffer, and FITC-dextran fluorescence was measured using an Aminco spectrofluorometer set at an excitation wavelength of 465 nm and an emission wavelength of 543 nm. Total FITC-dextran fluorescence in the sample was determined after treating the vesicles with 0.5% Triton X-100 to release all of the trapped fluorescent reporters. The percent leakage of FITC-dextran was determined as $\sim (F_t - F_0)*100/(F_T - F_0)$ where F_t is fluorescence (arbitrary units) of samples treated with PLA_2 for time t, F_0 is fluorescence of mock-treated samples, and F_T is total fluorescence determined after Triton X-100 permeabilization.

Chromatographic Enrichment of Flippase Activity—TE and TE-derived fractions were fractionated on DE52 and CM-Sepharose. Detergent (1% Triton X-100) was present in all buffers. Samples were dialyzed to adjust to buffer and salt conditions determined to be optimal for column binding. Samples to be fractionated on DE52 were adjusted to 20 mM Tris-HCl, pH 8.0, 25 mM NaCl, 1% Triton X-100; samples for CM-Sepharose were adjusted to 20 mM citrate buffer, pH 4.0, 25 mM NaCl, 1% Triton X-100. Binding was carried out in batch mode with end-over-end mixing for 2 h. Supernatant solutions containing unbound material were separated from the resin by centrifugation and tested for flippase activity after dialysis (to adjust salt to 100 mM) and reconstitution.

Flippase activity was calculated first by determining the extent, x, of [^{3}H]DPPC hydrolysis beyond that seen in liposomes, *i.e.* the amplitude of the second phase of the hydrolysis time course shown in Fig. 2*B*, and second, by normalizing the result to the protein/phospholipid ratio, r. Thus, specific activity = x/r. The total activity was calculated by multiplying the specific activity by the total amount of protein.

Analytical Methods—Phospholipids were quantitated by measuring lipid phosphorus (21). Protein content was determined with the micro-BCA protein assay reagent kit from Pierce Chemical Co. In the case of proteoliposomes, especially those with a low protein/phospholipid ratio, samples were delipidated, precipitated, and dissolved in 1% SDS before protein measurement (17, 18, 22). All lipid and protein estimations were performed in duplicate. All experiments were performed in duplicate unless mentioned otherwise. Proteins were analyzed by SDS-PAGE.

RESULTS

PLA_2-based Assay to Measure Transbilayer Movement of Phospholipids—The assay is illustrated in Fig. 1. Glycerophospholipids located in the outer leaflet of a membrane vesicle are susceptible to hydrolysis by exogenously added PLA_2. In the absence of any transbilayer transport as in liposomes, all phospholipids in the outer leaflet are expected to be converted to lysophospholipids and fatty acids by PLA_2, whereas phospho-

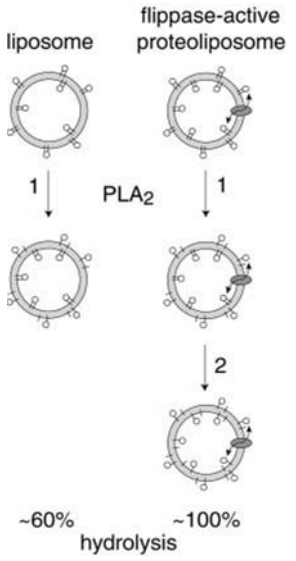

FIG. 1. **Flippase assay using reconstituted vesicle preparations and PLA_2.** Shown is a schematic representation of the assay. PLA_2 hydrolyzes the *sn*-2 ester of glycerophospholipids in the outer leaflet of membrane vesicles to yield lysophospholipid and fatty acid, products that are readily distinguished from intact phospholipids by TLC. In large unilamellar liposomes, PLA_2 would be expected to hydrolyze ~60% of the total phospholipid, *i.e.* the proportion of phospholipids residing in the outer leaflet. If the vesicles are equipped with a flippase capable of facilitating bidirectional phospholipid translocation, then 100% of the phospholipids will be hydrolyzed as inner leaflet lipids are flipped out and become accessible to PLA_2 (outer leaflet lysophospholipid + fatty acid complexes are presumed to be flipped in to compensate). Because lysophospholipids and fatty acids associate loosely, the membrane bilayer structure is preserved despite PLA_2 treatment, and the membrane permeability barrier is unaffected as seen by retention of fluid phase vesicle content markers.

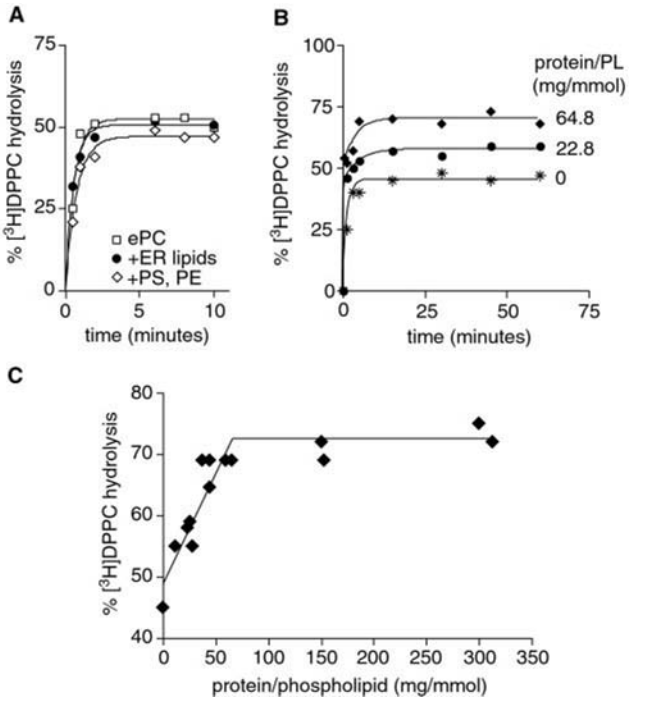

FIG. 2. **Extent of phospholipid hydrolysis in PLA_2-treated liposomes and proteoliposomes.** *A*, time course of [^{3}H]DPPC hydrolysis in PLA_2-treated liposomes prepared from mixtures of [^{3}H]DPPC and ePC (□), ePC supplemented with a Bligh-Dyer lipid extract corresponding to 100 μl of TE (●), and ePC supplemented with 5 mol% each of PS and PE (◇). The *lines* through the *points* correspond to single-exponential fits of the data; each *line* is characterized by a half-time of ~0.1 min and an amplitude of ~50%. *B*, time course of [^{3}H]DPPC hydrolysis in PLA_2-treated proteoliposomes prepared from [^{3}H]DPPC, ePC, and different amounts of TE (●, ♦). The protein/phospholipid (protein/PL) ratio of the proteoliposome samples is indicated. A sample of [^{3}H]DPPC/ePC liposomes (*) was assayed in parallel as a control. The time courses for both the proteoliposome samples are each best described by a double-exponential function (characterized by half-times of ~0.1 and ~3 min), whereas the *trace* for the liposome sample represents a single exponential fit as in *A*. *C*, effect of protein/phospholipid ratio on the maximum extent of [^{3}H]DPPC hydrolysis in PLA_2-treated proteoliposomes. The extent of hydrolysis increases from ~50% for protein-free liposomes to a maximum of ~75% for proteoliposomes prepared at a protein/phospholipid ratio of ≥ 60 mg/mmol.

lipids in the inner leaflet will be protected from hydrolysis. For unilamellar liposomes of ~250-nm diameter, up to 60% of the total phospholipid is expected to be hydrolyzed (23). If the vesicles contain a flippase capable of facilitating bidirectional translocation of phospholipids, then the extent of hydrolysis is expected to increase to 100% as inner leaflet lipids are flipped to the outer leaflet and become accessible to PLA_2 (outer leaflet lysophospholipids + fatty acid complexes are presumed to be flipped to the inner leaflet to compensate (24)). Because both products of PLA_2 cleavage (the lysophospholipid and fatty acid) remain in the membrane, the vesicles remain structurally intact and retain their permeability barrier (see below). Indeed, stoichiometric mixtures of lysophospholipids and fatty acids are known to form bilayer structures when dispersed in aqueous media, suggesting that despite the lack of covalent linkage, the lysophospholipid and fatty acid remain associated or pack together in such a way as to form a bilayer membrane (25).

Extent of PLA_2-mediated [^{3}H]DPPC Hydrolysis in Liposomes and Proteoliposomes—When ePC vesicles prepared with trace amounts of [^{3}H]DPPC were treated with PLA_2, roughly half of the [^{3}H]DPPC was converted to lyso- (monoacylated) [^{3}H]PC and palmitic acid in the outer leaflet (Fig. 2*A*, □). The time course of hydrolysis could be well described by a single exponential function, with a half-time of ~0.1 min to reach a plateau value of ~45–50% hydrolysis. Similar time courses were seen with ePC vesicles containing ER lipids (comparable with the amount of phospholipid in 100 μl of a TE of SWER (see below); Fig. 2*A*, ●), ePC vesicles containing a mixture of PS and PE (5 mol% each; Fig. 2*A*, ◇), and ePC vesicles that had been subjected to multiple freeze-thaw cycles, followed by extrusion through a 0.2-μm filter (data not shown). We conclude that the liposome preparations are unilamellar and that only those phospholipids located in the outer leaflet are accessible to PLA_2.

Similar experiments were carried out with proteoliposomes prepared from a solution of [^{3}H]DPPC/ePC supplemented with a TE of SWER vesicles. Unlike the time course of hydrolysis seen with liposomes, the time course of PLA_2-mediated hydrolysis of [^{3}H]DPPC in the proteoliposome samples showed biphasic behavior, reaching a final extent of hydrolysis greater than that seen with liposomes (Fig. 2*B*). For the two proteoliposome preparations analyzed in Fig. 2*B*, the final extent of [^{3}H]DPPC hydrolysis was greater in the sample prepared at a protein/phospholipid ratio of ~65 mg/mmol compared with the sample prepared at ~23 mg/mmol.

A systematic investigation of the extent of PLA_2-mediated [^{3}H]DPPC hydrolysis in proteoliposomes as a function of the protein/phospholipid ratio of the preparations is shown in Fig. 2*C*. The extent of hydrolysis increased linearly from ~45% for protein-free liposomes to ~75% for proteoliposomes prepared at a protein/phospholipid ratio of ~60 mg/mmol. This can be explained by noting that an increase in the protein/phospholipid ratio in the range of 0–60 mg/mmol increases the number of flippase-containing vesicles in the sample, *i.e.* at a low protein/phospholipid ratio not every proteoliposome contains a flippase, whereas at protein/phospholipid ratios approaching

60 mg/mmol the majority of proteoliposomes contain one flippase/vesicle on average. Thus the 0–60 mg/mmol regime is characterized by vesicles containing no or one flippase/vesicle, tending to zero flippases/vesicle at 0 mg/mmol and one flippase/vesicle at 60 mg/mmol.

No additional hydrolysis was seen when the protein/phospholipid ratio was increased beyond ~60 mg/mmol. The reason for hydrolysis plateauing at ~75% instead of 100% as suggested in the schematic in Fig. 1 is unclear (see "Discussion"). A maximum of 90–95% hydrolysis was achieved, however, when the membrane permeability barrier was disrupted by adding 0.5% Triton X-100 before the addition of PLA_2. We interpret the data shown in Fig. 2*C* as follows. We propose that for proteoliposomes with a protein/phospholipid ratio of ~60 mg/mmol, each vesicle in the preparation contains one flippase unit capable of transporting inner leaflet phospholipids to the outer leaflet where PLA_2-mediated hydrolysis to lysophospholipid and fatty acid occurs. Increases in protein/phospholipid ratio beyond ~60 mg/mmol result in vesicles with more than one flippase unit/vesicle on average. An increase in the number of transporters beyond one/vesicle does not change the amplitude of transport (monitored as the extent of PLA_2-mediated hydrolysis) as seen by the hydrolysis plateau in Fig. 2*C* but is expected to increase the rate of transport as discussed below.

Kinetics of PLA_2-mediated [^{3}H]DPPC Hydrolysis in Proteoliposomes—The biphasic time course of [^{3}H]DPPC hydrolysis in PLA_2-treated proteoliposomes consists of an initial phase similar in rate and extent to that seen with liposomes, followed by a slower second phase (Fig. 2*B*). The initial phase corresponds to the rapid hydrolysis of phospholipids situated in the outer leaflet of the vesicles and is independent of the protein/phospholipid ratio of the proteoliposome preparation (Fig. 3*A*). We argue that the second, slower phase corresponds to the rate-limiting transbilayer movement of [^{3}H]DPPC from the inner leaflet to the vesicle exterior, presumably accompanied by the compensatory inward flipping of PC and quasi-complexes of lyso-PC and fatty acid.

One prediction of this idea is that the second phase half-time will be independent of the protein/phospholipid ratio in the range of 0–60 mg/mmol in which proteoliposome preparations contain ≤ one flippase/vesicle as discussed above, but will decrease proportionately once the protein/phospholipid ratio exceeds 60 mg/mmol and preparations have > one flippase/vesicle on average. Fig. 3*B* shows that this is indeed the case. The transbilayer movement of [^{3}H]DPPC in vesicles equipped with a single flippase unit is characterized by a half-time of 3.3 min. As the protein/phospholipid ratio increases past 60 mg/mmol, the half-time of transbilayer translocation decreases. The slope of the line drawn in the >60 mg/mmol regime in Fig. 3*B* corresponds to a decrease in rate proportional to the increase in the protein/phospholipid ratio.

Proteolysis Reduces the Extent of [^{3}H]DPPC Hydrolysis in PLA_2-treated Proteoliposomes—The data presented in Figs. 2 and 3 suggest that specific membrane proteins in the TE are required to translocate [^{3}H]DPPC from the inner leaflet to the outer leaflet of proteoliposomes (see "Discussion"). To test this further, we investigated the effect of proteolysis on the extent of [^{3}H]DPPC hydrolysis in PLA_2-treated proteoliposomes. Proteoliposomes (protein/phospholipid ratio ~37 mg/mmol) were incubated with trypsin for 20 min at room temperature after which the trypsin was inactivated by the addition of trypsin inhibitor (a control sample was incubated with a combination of trypsin and trypsin inhibitor). The proteoliposomes were then assayed for their ability to translocate [^{3}H]DPPC. As shown in Fig. 4*A*, trypsin treatment resulted in a reduction in the number of flippase-active vesicles (seen as a reduction in the extent of [^{3}H]DPPC hydrolysis in the slow kinetic phase) by about 30% and a decrease in the rate of flipping (by a factor of ~1.7). Increasing amounts of trypsin caused progressively greater effects (Fig. 4*B*). These data confirm that transbilayer movement of [^{3}H]DPPC requires the participation of trypsin-sensitive membrane proteins.

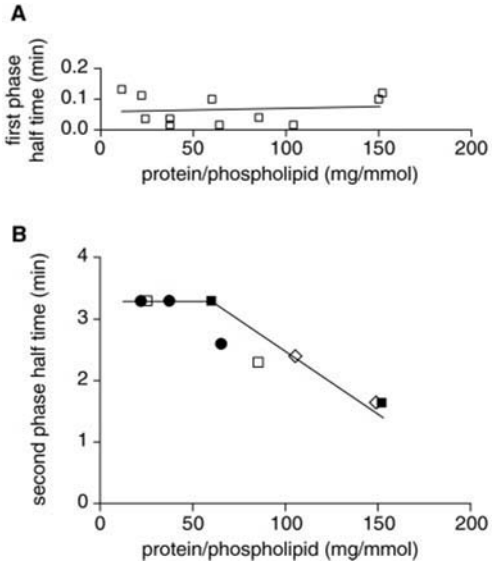

FIG. 3. **Kinetics of [^{3}H]DPPC hydrolysis in proteoliposomes.** *A* and *B*, data were collected from four independent experiments in which proteoliposomes, prepared at different protein/phospholipid ratios, were treated with PLA_2. In each case the time course of hydrolysis was fit to a double-exponential function. The graph in *A* shows the half-time for the first exponential phase, *i.e.* the first kinetic event during [^{3}H]DPPC hydrolysis by PLA_2 (see Fig. 1*A*), as a function of protein/phospholipid ratio. The *line* through the *points* represents a linear fit. The graph in *B* shows the half-time of the second exponential, *i.e.* the second kinetic event during [^{3}H]DPPC hydrolysis by PLA_2 (see Fig. 1*A*), as a function of the protein/phospholipid ratio. The different *symbols* correspond to different experimental sets of data, obtained on different occasions with different SWER preparations. Because of some variation among experimental sets, the sets were normalized to facilitate presentation in one graph. The half-times in the regime ≤60 mg/mmol ranged from 2 to 5.4 min, with an average of 3.3 ± 1.1 min (mean ± S.D.). Thus a single point in the ≤60 mg/mmol region of the plot for each of the data sets was set at 3.3 min, and the remainder of the data within the set were scaled accordingly. For example, for the data depicted by the *solid squares* (■) the second exponential phase half-time was determined to be 2 min and 1 min for the samples prepared at a protein/phospholipid ratio of 60 and 150 mg/mmol, respectively. These values were scaled to 3.3 and 1.65 min, respectively, for display in the graph in *B*. Similarly, for the experiment depicted by the *open squares* (□), the second exponential phase half-time was determined to be 5.4 and 3.8 min for samples prepared at 25 and 85 mg/mmol, respectively. These values were scaled to 3.3 and 2.3 min, respectively, for display in the graph in *B*.

Vesicle Intactness during PLA_2 Treatment—PLA_2 treatment results in hydrolysis of up to 75% of the phospholipid in proteoliposome preparations of ≥60 mg/mmol. To verify that the barrier function of the membrane is not compromised under these conditions and that PLA_2 does not have direct access to phospholipids in the inner leaflet of the vesicles, we tested the ability of the vesicles to retain soluble content markers that were trapped in the vesicle interior during reconstitution. In preliminary experiments we trapped [^{3}H]mannose in liposomes and proteoliposomes and measured the extent to which [^{3}H]mannose leaked of the vesicles in response to PLA_2 treatment for 60 min. Vesicles were centrifuged after PLA_2 treatment, and an aliquot of the supernatant was taken for liquid scintillation counting. The results showed that ~8–10% of the [^{3}H]mannose radioactivity in the vesicle sample was released and that this level of leakage occurred in both liposomes and proteoliposomes irrespective of PLA_2 treatment (result not shown).

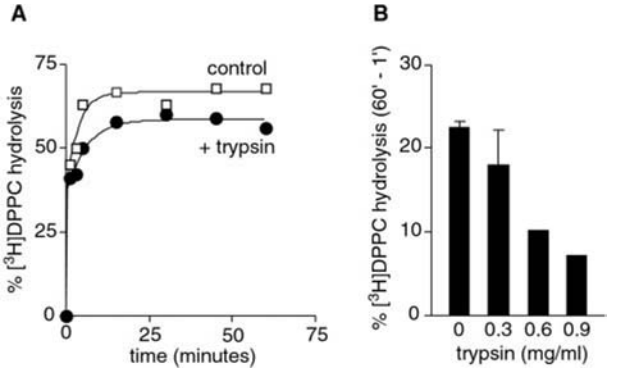

FIG. 4. **Protease sensitivity of PLA_2-mediated [^{3}H]DPPC hydrolysis in proteoliposomes.** *A*, effect of protease treatment on the extent of PLA_2-mediated [^{3}H]DPPC hydrolysis. Proteoliposomes (protein/phospholipid ratio ~37 mg/mmol) were incubated with a combination of trypsin and trypsin inhibitor (0.3 and 0.6 mg/ml, respectively) (control) or with 0.3 mg/ml trypsin for 30 min at room temperature before adding excess trypsin inhibitor to stop proteolysis. The vesicles were then treated with PLA_2 for different periods of time. The plot shows data points and double-exponential fits of the time course of [^{3}H]DPPC hydrolysis in control and trypsin-treated vesicles. For both plots the half-time and amplitude of the first exponential phase were ~0.04 min and ~36%, respectively. The second exponential phase was characterized by half-times of 2.4 min (control) and 4.2 min (trypsin-treated) and amplitudes of 30.6% (control) and 22.5% (trypsin-treated). *B*, effect of increased proteolysis. Proteoliposomes (protein/phospholipid ratio ~37 mg/mmol) were treated with different amounts of trypsin (0.3–0.9 mg/ml) before the extent of PLA_2-mediated hydrolysis in the second kinetic phase was assessed (see Fig. 1*A*). The extent of hydrolysis during the first kinetic phase was assessed at 1 min after the addition of PLA_2 (43.6 ± 2.0% (mean ± S.D.)), whereas total hydrolysis was assessed 60 min after the addition of PLA_2. The extent of PLA_2-mediated hydrolysis in the second kinetic phase was calculated as [^{3}H]DPPC hydrolysis at 60 min minus [^{3}H]DPPC hydrolysis at 1 min. The extent of PLA_2-mediated hydrolysis in the control (0 mg/ml trypsin) sample is an average obtained from samples in which proteoliposomes were incubated with buffer alone or with a combination of trypsin and trypsin inhibitor as in *A*.

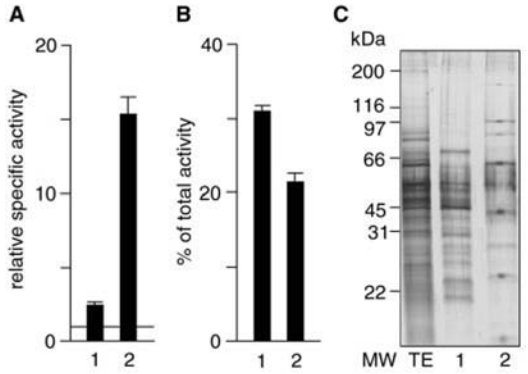

FIG. 5. **Fractionation of the TE and production of a protein fraction enriched in flippase activity.** TE (adjusted to 20 mM Tris-HCl, pH 8.0, 25 mM NaCl, 1% Triton X-100) was incubated with pre-equilibrated DE52 resin as described under "Experimental Procedures." The supernatant (fraction 1) was removed, adjusted to 20 mM citrate buffer, pH 4.0, 25 mM NaCl, 1% Triton X-100, and incubated with preequilibrated CM-Sepharose fast flow resin. The supernatant (fraction 2) was collected. Unfractionated TE and fractions 1 and 2 were reconstituted such that the resulting protein/phospholipid ratio was in the linear range of the PLA_2 assay (the TE sample, fraction 1, and fraction 2 were reconstituted at ~25, ~16, and ~1.5 mg/mmol, respectively). Flippase activity was calculated as described under "Experimental Procedures." *A*, enrichment of flippase activity in different fractions (normalized to the specific activity of the unfractionated TE set at 1 (indicated by the *horizontal line*). *B*, recovery of flippase activity normalized to the activity in the TE (set at 100%). *C*, silver-stained SDS-PAGE (12% acrylamide) gel of the TE (0.5 equivalent) and fractions 1 and 2 (1 equivalent each). The *numbers* on the *left* indicate the relative positions of molecular mass markers.

We confirmed this result using a different leakage assay in which FITC-dextran (molecular mass ~ 4,300 Da) was trapped at a self-quenching concentration within the vesicles. FITC-dextran-containing vesicles were treated with PLA_2 to measure [^{3}H]DPPC transport. Mock-treated samples were used as controls. Total FITC-dextran fluorescence in the sample was determined after treating the vesicles with 0.5% Triton X-100 to release all of the trapped fluorescent reporters. The results reinforced the data obtained with [^{3}H]mannose: there was no detectable leakage of FITC-dextran from liposomes and proteoliposomes treated for 20 min with PLA_2 and only a low level of leakage (6 ± 2% of the total, detergent-released signal) in vesicles treated for 60 min with PLA_2. Trypsin-treated vesicles, such as those described in Fig. 4, displayed identical characteristics (data not shown). We conclude that PLA_2 treatment does not disrupt the membrane permeability barrier in samples treated for 20 min, a time period more than sufficient to reach equilibrium in the transport/hydrolysis assays shown in Fig. 2, *A* and *B*.

Fractionation of the TE to Generate Fractions Enriched in Flippase Activity—As an initial step toward purifying a phospholipid flippase from rat liver ER, we chromatographed the TE on ion exchange resins. The TE was first incubated with preequilibrated DE52 anion exchange resin, and the unbound protein was incubated further with CM-Sepharose fast flow cation exchange resin. Incubations were carried out in batch mode. Unfractionated TE and samples of unbound material at both stages of incubation with the ion exchange resins were mixed with [^{3}H]DPPC and ePC and reconstituted into proteoliposomes. The proportion of each fraction taken for reconstitution was adjusted to yield proteoliposomes with a protein/phospholipid ratio in the linear range of the hydrolysis response. The specific activity of a particular sample was determined by normalizing the extent of "second-phase" hydrolysis to the protein/phospholipid ratio of the preparation.

Fig. 5*A* shows that flippase activity is enriched successively in TE samples passed sequentially over DE52 and CM-Sepharose resins (*A*), yielding a fraction ~15-fold enriched in flippase activity compared with the TE. The final recovery of activity was ~20% (Fig. 5*B*). Fig. 5*C* shows silver-stained SDS-PAGE protein profiles of unfractionated TE, material not bound to DE52, and material passed over both DE52 and CM-Sepharose. The simplification of the protein profile after successive incubations with the two resins is evident (Fig. 5*C*). These data indicate that the PLA_2-based assay can be used to monitor fractionation of the TE to generate simplified protein fractions enriched in flippase activity.

DISCUSSION

We describe procedures to measure the transbilayer translocation of DPPC in proteoliposomes reconstituted from rat liver ER. The principal features of the translocation assay are that (i) it utilizes a membrane-embedded "natural" phospholipid rather than a phospholipid analog as transport reporter; (ii) it allows measurement of the kinetics of transbilayer transport of a natural phospholipid; and (iii) it can be used to guide the purification of a flippase from a detergent extract of ER. Using the assay we were able to demonstrate the main conclusions of our previous work on an ER-derived flippase reconstitution system in which dibutyroyl-PC was used as the transport reporter (17) while importantly extending these earlier results to measure the kinetics of transbilayer transport.

Intactness of PLA_2-treated Vesicles Used in the Flippase Assay—PLA_2 is an interfacial enzyme that operates in either a highly processive, vesicle-associated scooting mode and/or a less processive hopping mode in which it can dissociate from one vesicle and associate with another (26, 27). Our assay conditions

ensure that regardless of whether hopping occurs appreciably, the ratio of vesicles to PLA_2 is such that each vesicle is likely to have at least one bound PLA_2 molecule. Once associated with the vesicle surface, PLA_2 hydrolyzes outer leaflet phospholipids rapidly, causing the hydrolysis of ~45% of the total phospholipid with a half-time of ~0.1 min (Fig. 2*A*). This is the result seen with liposomes prepared from ePC, or mixtures of ePC with other lipids, consistent with previous reports (28, 29).

A potential concern with the assay is that the massive hydrolysis of vesicle phospholipids caused by PLA_2 treatment (Fig. 2) could promote phospholipid flip-flop as well as vesicle disintegration. PC may be viewed as a cylindrically shaped phospholipid, whereas lyso-PC may be regarded as an inverted cone (30), the presence of which may create molecular packing defects leading to increased flip-flop and bilayer collapse. The limited hydrolysis seen with liposomes (maximum 45%, corresponding well with the proportion of phospholipids located in the outer, PLA_2-accessible monolayer; see below for a detailed discussion of the extent of hydrolysis), as well as the protease susceptibility of flipping seen in proteoliposomes argue strongly against the idea that lyso-PC causes flipping in the reconstituted vesicle system. Our experimental tests of the retention of trapped [^{3}H]mannose and FITC-dextran in PLA_2-treated liposomes and proteoliposomes strongly suggest that PLA_2-treated vesicles remain intact under our experimental conditions. Similar observations have been reported previously in studies of PLA_2-treated mitochondrial outer membrane vesicles (31).

A plausible explanation for the noneffect of lysophospholipid on flipping and membrane integrity is that the vesicles contain the other product of PLA_2 hydrolysis, namely free fatty acid. Geometric considerations dictate that the presence of a fatty acid adjacent to a lysophospholipid molecule would correct any molecular packing defect caused by the lysophospholipid alone because the quasi-complex of lysophospholipid and fatty acid would have a bilayer-promoting cylindrical shape. Indeed, work by Jain and co-workers (25) demonstrated that a stoichiometric mixture of fatty acid and lyso-PC forms a membrane bilayer structure even though the individual components form micelles when dispersed in aqueous media. These results, together with other studies showing that liposomes maintain their integrity even when their outer monolayer of phospholipids is completely degraded by PLA_2 but collapse when outer leaflet fatty acids are extracted by bovine serum albumin (29, 32), support the idea that the simultaneous presence of both products of PLA_2 hydrolysis preserves the membrane barrier function.

Extent of Hydrolysis by PLA_2—The maximum hydrolysis achieved by PLA_2 treatment of proteoliposomes was ~75% (Fig. 2*C*). As described under "Results" and discussed briefly below, we argue that proteoliposome preparations in which maximal hydrolysis is achieved contain ≥ one flippase/vesicle. In such vesicles, both PC and lyso-PC-fatty acid complexes would have access to both leaflets of the vesicle membrane, and hydrolysis would be expected to go to completion as suggested in the schematic in Fig. 1. The reason for the incomplete hydrolysis is unclear, but it may have to do with a number of factors, including product inhibition caused by accumulation of lysophospholipids and fatty acids in the outer leaflet, changes in surface potential accompanying product formation (27), limited phase separation of lysophospholipids and fatty acids away from intact phospholipids (32), and the potentially limiting extent of compensatory inward movement of lysophospholipids and fatty acids resulting from the preference of lysophospholipids for the convex outer monolayer of the vesicles (28–30).

Although these ideas are partly speculative, they form the basis for rationalizing the limited hydrolysis seen in liposomes and matching this to the hydrolysis seen in flippase-equipped proteoliposomes. Light scattering measurements indicate that the liposomes and proteoliposomes in our experiments have an average diameter of ~250 nm, from which it can be deduced that ~60% of the phospholipids are located in the outer monolayer and ~40% in the inner monolayer. PLA_2 treatment of liposomes results in hydrolysis of 45% of the total phospholipid, *i.e.* ~75% of the outer leaflet phospholipid is converted to lyso-PC on PLA_2 treatment, with ~25% of the PC remaining intact. If it is assumed that once this surface proportion of intact phospholipid and lysophsopholipid/fatty acid is reached no further hydrolysis is possible for the reasons suggested above, then it can be deduced that in PLA_2-treated, flippase-equipped proteoliposomes the outer leaflet would contain 45% lyso-PC + 15% PC, and the inner leaflet would contain 30% lyso-PC + 10% PC (percentages of total phospholipid). Thus total hydrolysis in proteoliposomes would be 75%, which fits with our experimental observations.

Abundance of the Flippase Relative to Reconstituted ER Membrane Proteins—The dose-response plot in Fig. 2*C* can be used to calculate the abundance of the flippase among detergent-solubilized and reconstituted ER membrane proteins. At the inflection point (~60 mg/mmol) of the dose-response plot, each proteoliposome is expected to contain a single flippase on average: for preparations in the range of 0–60 mg/mmol, vesicles contain either no or one flippase/vesicle, whereas for samples prepared at >60 mg/mmol, vesicles contain one or more flippases/vesicle on average. Liposomes and proteoliposomes with an average diameter of ~250 nm contain ~550,000 phospholipids/vesicle[3] (23, 33). Using this figure and assuming that the mean molecular mass of ER membrane proteins is ~50 kDa (based on analyses of baby hamster kidney cell ER preparations; see Ref. 34), each vesicle prepared at ~60 mg/mmol contains ~660 protein molecules on average. This implies that the abundance of functional flippases is about 1 in 660 or ~0.15% by weight of reconstituted ER membrane proteins. This estimate is very similar to the 0.2% figure we deduced in our work using dibutyroyl-PC as transport reporter (17), as well as the estimate of 0.6% obtained in an earlier study (35).

Kinetics of Transbilayer Transport—The biphasic time courses of PLA_2-mediated DPPC hydrolysis in proteoliposomes provide an opportunity to assess the rate of phospholipid translocation. As described under "Results," the initial phase of hydrolysis can be characterized as a burst, representing rapid hydrolysis of outer leaflet phospholipids by PLA_2. The second, slower phase is dictated by the rate-limiting translocation of phospholipids from the inner to the outer leaflet and the likely compensatory inward translocation of lysophospholipids and fatty acids. For proteoliposomes prepared at a protein/phospholipid ratio of ≤ 60 mg/mmol, *i.e.* containing a single functional flippase/vesicle, the characteristic half-time of the second hydrolysis phase was determined to be 3.3 min (Fig. 3*B*). Thus, roughly 165,000 phospholipid molecules[4] are translocated across the bilayer in each direction by a single functional flip-

[3] The proteoliposomes in our preparations were determined to have an average diameter of 250 nm by light scattering analysis. Assuming a bilayer thickness of ~4 nm and a phospholipid cross-sectional area of ~0.7 nm^2, it can be calculated that each proteoliposome has ~550,000 phospholipids on average, with ~330,000 in the outer leaflet and ~220,000 in the inner leaflet (23, 33).

[4] Approximately 45% of the total phospholipid is hydrolyzed in the initial burst seen after the addition of PLA_2, *i.e.* 245,000 molecules representing ~75% of the outer leaflet pool. To reach a maximum hydrolysis of ~75% as seen in proteoliposomes with a protein/phospholipid ratio of 60 mg/mmol, ~165,000 phospholipids (~30% of the total phospholipid in the vesicle) must be flipped out (externalized) from the inner leaflet with the compensatory internalization of ~165,000 phospholipids and quasi-complexes of lysophospholipid and fatty acid.

pase, with total mixing being achieved with a half-time of 3.3 min. For vesicles populated with more than one functional flippase, the half-time of inner and outer monolayer mixing drops proportionately (Fig. 3*B*). The transport half-time measured in these experiments corresponds reasonably well with the half-times measured for the translocation of acyl-NBD phospholipid analogs in proteoliposomes generated from detergent extracts of *Escherichia coli* (12). In a similar limiting regime in which the vesicles were prepared such that they contained only a single flippase, the half-time for translocation was determined to be ~30–60 s. This faster rate could be a characteristic of the short chain acyl-NBD phospholipid analogs used as transport reporters; alternatively the slower times measured with the present assay could be the result of constraints created by the accumulation of hydrolysis products.

It is interesting to use these data to consider the translocation process. A rough calculation based on the Berg and Purcell concept of a "mean time to capture" (36) of a phospholipid that diffuses freely until it encounters a flippase indicates that in proteoliposomes containing one immobile flippase the capture frequency is ~1.5 phospholipids/μs.[5] Thus, if the flippase is a perfect absorber and the transbilayer translocation step is not rate-limiting, transport of ~165,000 phospholipid molecules from the inner leaflet to the outer leaflet would be achieved in ~40 ms, 3 orders of magnitude more rapidly than the experimentally measured time frame of 3.3 min. Comparison of this result with the experimental data indicates that the slow transport step is flippase-mediated phospholipid translocation across the bilayer, not capture of a phospholipid by the flippase.

Is the flipping rate measured in our assay compatible with the rate of phospholipid synthesis and ER bilayer propagation in a living cell? Based on the experimentally determined rate of PC synthesis in hepatocytes (42) and the assumptions implicit in our calculation of flippase abundance relative to other ER membrane proteins (see above), it can be calculated[6] that the cellular requirement for flipping is ~10 molecules of PC flipped/min/flippase in the ER. If the flippase translocates all phospholipids irrespective of headgroup (6–8), then the flipping requirement would be 20–30 phospholipid molecules/min/flippase because PC represents ~40–60 mol% of ER phospholipids (23, 43). The phospholipid transbilayer equilibration rate measured here indicates that phospholipid translocation mediated by a single flippase occurs at a rate on the order of 10^4 molecules/min, more than adequate for ER bilayer propagation in a living cell.

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[5] The mean time to capture, t_{dc}, of a particle (phospholipid) diffusing on a spherical surface until it hits a fixed absorbing region is $t_{dc} = (b^2/2D)(\ln(b/s)-1)$ (see Refs. 36–38), where the flippase is modeled as an immobile absorbing region of radius $s = 10^{-7}$ cm (modeled loosely on the radius of a magainin pore, which mediates phospholipid flip-flop while permitting transport of small solutes (39)); b corresponds to the diameter of the sphere (~2.5×10^{-5} cm), and D is the phospholipid diffusion coefficient (10^{-8} cm^2/s) (40, 41). The expression for t_{dc} holds when $1 \gg (s/b)2$ which is the case here. Using the given numbers, t_{dc} can be calculated as ~ 0.14 s. If the flippase is a perfect absorber and depletes the phospholipid inner monolayer exponentially, then transport of ~165,000 phospholipids of 220,000 in the inner monolayer would take ~0.14(−ln(1.65/2.2)) or ~ 0.04 s.

[6] The rate of PC synthesis in hepatocytes is ~3.7 nmol/min/g, wet weight, of cells (41). Our data indicate that the yield of SWER is 6.7 mg/g, wet weight, of liver. Thus PC synthesis is 0.55 μmol/min/g of SWER; if 50% of the PC molecules need to be flipped to disperse the molecules evenly in both leaflets of the bilayer, the flipping rate is ~0.27 μmol/min/g of SWER. Assuming that the average molecular mass of ER membrane proteins is 50 kDa, and the flippase represents 0.15% by mass of ER membrane proteins (see "Discussion"), the flipping rate can be rewritten as ~10 PC molecules flipped/min/flippase.