Flippase Activity in Proteoliposomes Reconstituted with *Spinacea oleracea* Endoplasmic Reticulum Membrane Proteins: Evidence of Biogenic Membrane Flippase in Plants[†]

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ABSTRACT: Phospholipid translocation (flip-flop) in biogenic (self-synthesizing) membranes such as the endoplasmic reticulum of eukaryotic cells (rat liver) and bacterial cytoplasmic membranes is a fundamental step in membrane biogenesis. It is known that flip-flop in these membranes occurs without a metabolic energy requirement, bidirectionally with no specificity for phospholipid headgroup. In this study, we demonstrate for the first time ATP-independent flippase activity in endoplasmic reticulum membranes of plants using spinach as a model system. For this, we generated proteoliposomes from a Triton X-100 extract of endoplasmic reticulum membranes of spinach and assayed them for flippase activity using fluorescently labeled phospholipids. The half-time for flipping was found to be 0.7−1.0 min. We also show that (a) proteoliposomes can flip fluorescently labeled analogues of phosphatidylcholine and phosphatidylethanolamine, (b) flipping activity is protein-mediated, (c) more than one class of lipid translocator (flippase) is present in spinach membranes, based on the sensitivity to protease and protein-modifying reagents, and (d) translocation of PC and PE is affected differently upon treatment with protease and protein-modifying reagents. Ca^{2+}-dependent scrambling activity was not observed in the vesicles reconstituted from plant ER membranes, ruling out the possibility of the involvement of scramblase in translocation of phospholipids. These results suggest the existence of biogenic membrane flippases in plants and that the mechanism of membrane biogenesis is similar to that found in animals.

The transbilayer movement (flip-flop) of phospholipids (PLs)[1] and other membrane lipids is fundamental to cellular membrane biogenesis and cell growth (*1, 2*). PL-synthesizing enzymes have their active sites oriented toward the cytosolic membrane half of the endoplasmic reticulum (ER) in eukaryotic cells and bacterial cytosolic membranes, which are collectively termed biogenic (self-synthesizing) membranes (*3*). The membrane PL synthesis and the distribution to various organelles are poorly understood in plants. In plants, the synthesis of membrane lipids is more complex than that in bacteria and animals and occurs in the ER and chloroplast (*4*). The fatty acyl groups are synthesized by enzymes present in the chloroplast and, after being converted to acyl-CoA, move to the ER and are utilized in the synthesis of all membrane phospholipids like phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and free sterols, which also exist in the ER (*4, 5*). The bulk of the phospholipids and sterols synthesized in the ER move to other organelles for their membrane biogenesis and also provide chloroplasts with acyl−glycerol back bones for the synthesis of the bulk of the thylakoid lipids (*5, 6*). The membrane lipids are synthesized in the cytosolic leaflet of the ER and stroma of plastids (*7, 8*). Therefore, ∼50% of the newly synthesized lipids are to be translocated to the luminal half-membrane of these organelles to ensure the uniform growth of these membranes.

Translocation of the polar head groups of PLs through the hydrophobic core of membrane bilayer is energetically unfavorable and very slow (halt-time varies from hours to days) in artificial membrane vesicles (*9*), but the half-time for translocation is very rapid (30 s to 3−5 min) in biogenic membranes (*10*−*18*). Researchers have also observed that PL translocation is rapid, bidirectional, and nonspecific to PL headgroup and does not require any metabolic energy. Interestingly, this rapid translocation also occurs only due to the presence of specific membrane proteins (*15*−*17*). Therefore, it has been proposed that biogenic membranes must contain proteins that facilitate a passive equilibration of newly synthesized lipids between the two membrane halves termed biogenic membrane flippase (*16, 17*). Apart from glycerophospholipids, a dedicated flippase is thought to be involved in the flipping of complex glycophospholipids

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[1] Abbreviations: PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; ePC, egg phosphatidylcholine; ER, endoplasmic reticulum; PM, plasma membrane; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PPR, protein/phospholipid ratio of reconstituted vesicles (milligrams per millimole); TE, Triton X-100 extract of spinach microsomes; BTP, 1,3-bis[tris(hydroxymethyl)methylamino]propane; NEM, *N*-ethylmaleimide; SWER, salt-washed endoplasmic reticulum; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DEPC, diethyl pyrocarbonate; PG, phenyl glyoxal; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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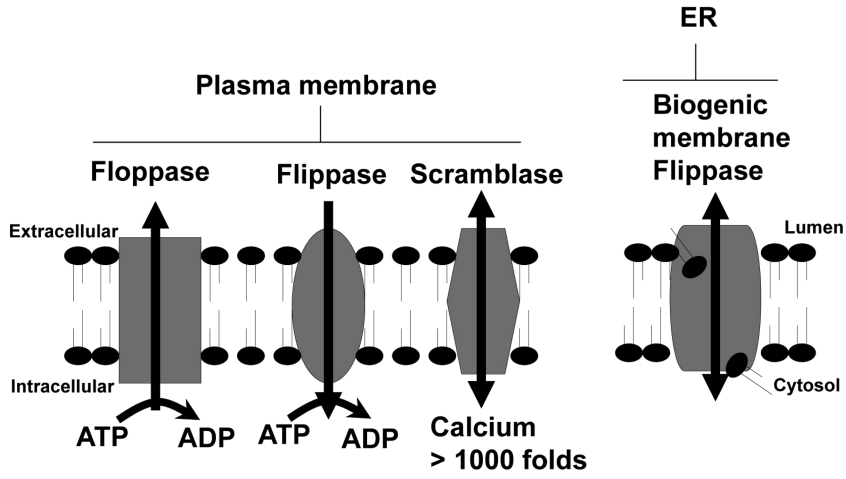

FIGURE 1: Overview of different phospholipid translocators present in cellular membranes. Outward-directed floppase translocates phosphatidylcholine and sphingomyelin from the inner leaflet of the plasma membrane to the outer leaflet, whereas the inwardly directed flippase (aminophospholipid translocator) translocates PS and PE from the outer leaflet to the inner leaflet. Phospholipid scramblase destroys the membrane asymmetry by rapidly and nonspecifically translocating the membrane phospholipid bidirectionally with a 1000-fold increase in the cytosolic calcium concentration. Biogenic membrane flippase translocates phospholipids bidirectionally, in a manner nonspecific to phospholipid headgroup without the requirement of metabolic energy (ATP).

such as dolicholphosphate hexoses during the synthesis of complex glycolipids and glycoproteins (*19*). The presence of a biogenic membrane flippase has been hypothesized in animals (e.g., rat liver ER) and BCM (e.g., *Bacillus megaterium*) membrane vesicles (*13*−*15*). As the synthesis and distribution of all membrane lipids in plants occur in ER and thylakoid membranes of chloroplasts, we hypothesize the presence of a biogenic membrane flippase in these membranes. As the biogenic membrane flippase is supposed to be a fundamental protein aiding in membrane biogenesis and considered to be conserved over the course of molecular evolution, we investigated the flippase activity in the plant ER membranes using spinach (*Spinacea oleracea* L. cv. New Asia) as a model system.

To date, various PL translocators have been purified and characterized. PL translocators can be classified into ATP-dependent and ATP-independent groups (Figure 1) (*20*). ATP-dependent translocators are localized in the plasma membrane (PM) of eukaryotic cells and are involved in maintaining PL asymmetry. This class of transporters is further classified into two types, viz., flippases, which are involved in vectorial transport of aminophospholipids from the exoplasmic side to the cytosol at the expense of ATP (e.g., aminophospholipid translocase), and floppases, which are involved in translocation of PLs from the cytosolic side to the outside (e.g., multidrug resistance proteins). In addition, PM is equipped with another class of translocators named scramblases, which scramble PLs across the membrane with a 1000-fold increase in the cytosolic calcium concentration (*20, 21*). However, no biogenic membrane flippases have been identified. Additionally, to date no evidence for the existence of biogenic membrane flippase activity in plants has been presented.

In this study, we prepared the Triton X-100 extract of the salt-washed endoplasmic reticulum (SWER) from the leaves of spinach and reconstituted the integral membrane proteins into proteoliposomes. Fluorescent lipid analogues of PC and PE were used as probes to study the PL translocation in spinach ER membranes. Our results clearly show that flippase activity was protein-mediated and sensitive to protease (trypsin) and several protein-modifying reagents. Our study also shows that the translocation of fluorescent lipid analogues in the spinach ER membrane is not due to the presence of a Ca^{2+}-dependent scramblase.

EXPERIMENTAL PROCEDURES

Materials. Egg phosphatidylcholine (ePC), sodium dithionite, diethyl pyrocarbonate (DEPC), bovine pancreatic trypsin, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), phenyl glyoxal (PG), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), ULTROL-grade Triton X-100, the protein estimation kit (BCA kit), cytochrome *c*, and routine chemicals were obtained from Sigma. SM-2 Biobeads were from Bio-Rad Laboratories. 1-Oleoyl-2-[6-(7-nitrol-2-1,3-benzonadiol-4-yl)-amino]-*sn*-glycerophosphocholine (NBD-PC) and 1-oleoyl-2-[6-(7-nitrol-2-1,3-benzonadiol-4-yl)amino]-*sn*-glycerophosphoethanolamine (NBD-PE) were from Avanti Polar Lipids.

Isolation of ER Microsomes from Spinach Leaves. The microsomal membrane fraction was prepared as described previously (*22*). Briefly, 100 g of fresh leaves was homogenized in 100 mL of MOPS-BTP buffer (pH 7.5) containing 0.33 M sucrose, 5 mM Na^{2+}-EDTA, 5 mm DTT, 0.2% (w/v) BSA, 1 mM benzamidine-HCl, and 0.5 mM PMSF. The homogenate was filtered through nylon cloth and centrifuged at 10000*g* for 15 min at 4 °C. The supernatant was centrifuged at 40000*g* for 1 h at 4 °C. The resulting pellet was suspended with a Teflon homogenizer in buffer A [25 mM MOPS-BTP (pH 7.5) and 0.33 M sucrose] supplemented with 0.5 M NaCl, 1 mM Na^{2+}-EDTA, 5 mM DTT, and 0.5 mM PMSF to a final volume of 25 mL and was again pelleted at 100000*g* for 45 min at 4 °C. The final washed microsomal pellet was suspended to ∼3 mL with buffer A with 5 mM DTT and 0.5 mM PMSF. The membranes were frozen in liquid nitrogen and stored at −80 °C for later use.

Purity of Isolated ER Membranes. Different subcellular fractions collected from the spinach ER were analyzed for cytochrome *c* reductase (CCR) activity, which is a marker protein for the ER according to the protocol described previously (*23*). Briefly, 50 μL of the purified fraction was added to 1 mL of assay mixture containing 300 mM

potassium phosphate (pH 7.8), 0.1 mM EDTA, 0.5 mg/mL BSA, 36.0 μM cytochrome *c*, and 0.85 mg/mL NADPH. The cytochrome *c* reductase activity was monitored spectrophotometrically by measuring the change in absorbance at 550 nm for 5 min with a Perkin-Elmer spectrophotometer using a kinetic model. One unit of enzyme activity was defined as 1 μmol of reduced cytochrome *c* formed, and its concentration was calculated by using an extinction coefficient of 21.1 M^{-1} cm^{-1} (*23*).

Preparation of Triton X-100 Extract (TE) from the Salt-Washed ER (SWER). The SWER was prepared from the spinach (*S. oleracea*) homogenate as previously described (*16*). Briefly, 3.0 mL of frozen (−80 °C) membranes was thawed on ice, and the total protein was estimated by the bicinchonic acid (BCA) method. The initial protein concentration was found to be 4.5 mg/mL. TE was prepared by first diluting the SWER suspension with an equal volume of 10 mM HEPES/NaOH buffer (pH 7.5) containing 100 mM NaCl and 0.1% (w/v) Triton X-100. The sample was mixed, left on ice for 30 min, and then ultracentrifuged (100 Ti rotor at 150000*g* for 30 min at 4 °C). The supernatant was carefully removed, and the pellet was resuspended to the original volume of the SWER in 10 mM HEPES/NaOH (pH 7.5), 100 mM NaCl, and 1% (w/v) Triton X-100. The sample was thoroughly mixed by being gently vortexed and/or pipetted and left on ice for 30 min before being ultracentrifuged as stated previously. The resulting clear supernatant, or Triton extract (TE), was used directly for reconstitution at a protein concentration of ~3.0 mg/mL.

Reconstitution of Liposomes and Proteoliposomes. The method of preparation of liposomes and proteoliposomes was followed as described previously (*15, 16*). ePC (4.5 μmol) and trace quantities (0.3 mol %) of fluorescent phospholipids (NBD-PC and NBD-PE) were dried under a stream of nitrogen and solubilized in 10 mM HEPES/NaOH (pH 7.5) and 100 mM NaCl containing 1% (w/v) Triton X-100. For the preparation of proteoliposomes, TE with known protein concentrations was added to the solubilized lipid samples. The Triton X-100 was removed with SM2 Biobeads, yielding the lipid vesicles as described previously (*24*). The liposomes were collected by ultracentrifugation (type 100 Ti at 150000*g* for 1 h at 4 °C) of the reconstituted sample and washed three times with 10 mM HEPES/NaOH (pH 7.5) and 100 mM NaCl to remove the non-reconstituted background fluorescent lipids and proteins. The amount of TE used was varied as required to prepare proteoliposomes with different protein/phospholipid ratios. To make lipid vesicles of uniform diameter (100 nm), the vesicles were passed several times (~15 times) through a 0.1 μm polycarbonate membrane filter using a lipid mini extruder (Avanti Poalr Lipids, Inc.). The protein/phospholipid ratio (PPR) of proteoliposome samples was determined as described previously (*25, 26*). The average error was <5% for protein measurements and <3% for phospholipid measurements.

Sucrose Floatation Gradient Analysis. A sucrose floatation gradient analysis was performed to verify the reconstitution of membrane proteins from TE as described previously (*27*). Briefly, 1 mL of the reconstituted proteoliposomes was layered under a series of sucrose steps [20, 10, 5, and 2.5% (w/w)] and centrifuged at ~100000*g* in a swinging-out rotor (SW 41-Ti) for 1 h. Fractions were collected from different sucrose interfaces and dialyzed against assay buffer for 24 h with several buffer changes to remove the sucrose from the fractions. The fractions were then analyzed for the estimation of protein and phospholipids as described previously (*25, 26*).

Assay for Flippase Activity. The assay was performed using a JASCO spectrofluorometer. A total of 50 μL of liposomes or proteoliposomes containing NBD-PL was added to 2.95 mL of 10 mM HEPES (pH 7.5) and 100 mM NaCl in a fluorescence cuvette. Time-dependent fluorescence (excitation at 470 nm, emission at 532 nm) was monitored continuously at 22 °C with constant low-speed stirring. After the fluorescence intensity was stabilized (~200 s), 6 μL of 1 M sodium dithionite (freshly prepared in 1 M Tris base) was added. When the fluorescence reached a second plateau (~400 s), 50 μL of 10% (w/v) Triton X-100 was added to completely permeabilize the vesicles. The initial fluorescence (F_0) was taken as the average value of the fluorescence intensity of the first plateau; the fluorescence after dithionite reduction (F_t) was taken as the average value of the second plateau. The percent of NBD-PL that was reduced was calculated by normalizing the F_0 to 1.0 and F_t as the fraction of F_0. The activity (*A*) of flippase in a proteoliposome preparation was taken as the difference between the percent reduced in the sample and the percent reduced in a liposome sample (control). Specific activity was defined as *A* divided by the protein/phospholipid ratio (in milligrams per millimole). The functional flippase activity of the proteoliposomes after their treatment with protein-modifying agents was calculated as the percentage of untreated control proteoliposomes as described previously (*15, 16*). Briefly

$$\mathrm{PFR} = 100(1 - F_t/F_0)$$

where PFR is the percentage fluorescence reduction

$$A = P_{\mathrm{PL}} - P_0$$

where P_{PL} is the percentage reduction of fluorescence intensity in the proteoliposomes and P_0 is the percentage reduction in control liposomes

$$\%\ \text{inhibition by the protein-modifying agent} = [1 - (A_{\text{protein-modifying agent}} - A_{\text{mock}})] \times 100$$

Treatment of Proteoliposomes with Protein Modification. The intact liposomes and proteoliposomes were treated with bovine pancreatic trypsin as described previously (*10, 15*). Briefly, 50 μL of liposomes (control) or proteoliposomes was added with desired concentrations of trypsin in 10 mM HEPES (pH 7.5) and 100 mM NaCl in a 200 μL reaction mixture and incubated at 37 °C for 30 min. The sample was then added with 2.8 mL of 10 mM HEPES (pH 7.5) and 100 mM NaCl, and the fluorescence intensity was monitored as described previously. For chemical modification of proteoliposomes, freshly prepared stock solutions of DEPC, DTNB, PG, and AEBSF [typically 50 mM reagent in 10 mM HEPES (pH 7.5) and 100 mM NaCl] were added to the liposome (control) or proteoliposome to yield the desired final concentration of protein modification reagent. The pH of the reaction was confirmed by pH paper to be 7.5. The number of free sulfhydryl groups in TE was measured using Ellman's reagent to ensure that all experiments utilizing DEPC and DTNB involved an excess of reagent over available SH groups. For all experiments with protein-modifying reagents, the modifiers were first titrated with a known amount of proteoliposomes to determine the effective

working concentrations of these reagents. Then the proteoliposomes with a known quantity of protein that is unsaturated for flippase activity were used to study the effect of these compounds on flippase activity (*16*). In all experiments, the integrity of the vesicles was determined by treating the control liposomes with the largest amount of reagent used in the experiment and monitoring the fluorescence intensity for 400 s after dithionite treatment and then treatment with Triton X-100.

Analysis of the Scramblase Activity Present in the ER Membrane Fraction. The measurement of scramblase activity was performed as described previously (*21*) with slight modification. Briefly, symmetrically labeled proteoliposomes were prepared as described in the previous section. Then these vesicles were treated with 20 mM dithionite for 5 min to quench the fluorescent lipid molecules of the outer leaflet and immediately centrifuged for 15 min at 400000*g* to pellet down the inside-labeled vesicles. Proteoliposomes containing NBD-PC or NBD-PE were incubated for 2 h at 37 °C in 10 mM HEPES/NaOH (pH 7.5) and 100 mM NaCl in presence of 2 mM Ca^{2+} or 4 mM EGTA and then transferred to a stirred fluorescence cuvette at 25 °C. The initial fluorescence was recorded in a spectrofluorometer for 100 s, and 20 mM dithionite was added. The difference between the nonquenchable fluorescence observed in the presence and absence of Ca^{2+} is contributed to the Ca^{2+}-induced scrambling of NBD-PC or NBD-PE located in the inner leaflet of proteoliposomes. A similar procedure was followed for symmetrically labeled vesicles.

RESULTS AND DISCUSSION

Flippase Assay Using Fluorescent PL Analogues. The flipping activity was measured by quenching of fluorescently labeled PLs by the membrane impermeable reagent sodium dithionite (Figure 2). In this study, we used 1-oleoyl-2-[6-(7-nitrol-2-1,3-benzonadiol-4-yl)amino]-*sn*-glycerophosphocholine (NBD-PC) and NBD-PE as fluorescent probes to measure the flippase activity. Liposomes and proteoliposomes were treated with sodium dithionite, and the degree of fluorescence quenching was measured as a function of time. In the case of liposomes or proteoliposomes which do not have flippase activity, all the PLs on the outer leaflet were quenched when they were treated with dithionite and yielded ~50% quenching, whereas inner leaflet PLs were protected from dithionite. Upon addition of 0.1% (w/v) Triton X-100 to liposomes, ~100% quenching was observed, indicating that the membrane permeability was disturbed leaving all the PLs accessible to dithionite (Figure 2). In the case of proteoliposomes (i.e., liposomes reconstituted with biogenic membrane flippases), NBD-PLs could equilibrate between the membrane leaflets; as a result, more than 50% quenching was observed (Figure 2). The fluorescence quenching of liposomes and proteoliposomes could be fitted to single-exponential and double-exponential decay functions, respectively, to calculate the rate of flipping and half-life of translocation[2] (Figure 2).

Isolation of the Spinach ER and Biochemical Reconstitution of Proteoliposomes from the Detergent Extract of SWER Membranes. Biogenic membrane flippase activity has been demonstrated in mammals, yeast, Gram-positive bacteria, and Gram-negative bacteria (*12*−*14*). However, so far, no

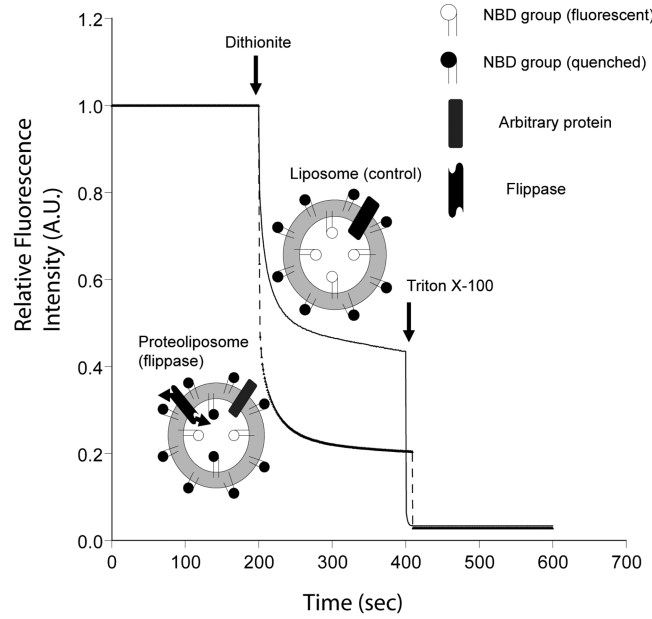

FIGURE 2: Schematic representation of the assay for flippase activity. Liposomes or proteoliposomes (50 μL) were added to 2.95 mL of assay buffer [10 mM HEPES/NaOH (pH 7.5) and 100 mM NaCl] with continuous stirring, and time-dependent fluorescence was monitored in a Jasco spectrofluorimeter for 10 min. All measurements were carried out at 25 °C under minimum light conditions (excitation at 470 nm, emission at 530 nm, response time of 1 s).

biogenic membrane flippase has been purified and characterized. Since the functions of flippases are more fundamental to the cell, we hypothesize that this activity should be conserved in all organisms. However, to date, no evidence of biogenic membrane flippase activity in plants has been found. Understanding membrane biogenesis in plants is very important for understanding the mechanism of membrane biogenesis and trafficking of membrane lipids and proteins to their target destinations inside the cell (*4*). For this purpose, we selected spinach as a model system for isolating ER membranes and checked biogenic membrane flippase activity. We isolated ER membranes from the spinach leaves as described previously and confirmed the purity of the isolated ER microsomes by measuring the activity of cytochrome *c* reductase (CCR), a marker enzyme for the plant ER membrane protein (*23, 28*). The results clearly showed that the CCR activity was enriched in the ER membrane fraction when compared to those of the crude lysate and other fractions (Figure 3A). These results strongly suggest that the membrane that we isolated was pure ER membrane and not contaminated with other membranes.

To enhance the flippase activity, we performed differential detergent extraction of the ER microsomes with Triton X-100. Initially, ER membranes were solubilized in buffer containing 0.05% (w/v) Triton X-100 to remove the periph-

[2] The double-exponential function is given by $F(t) = F_0 - [A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t)]$, where $F(t)$ is the fluorescence as a function of time, F_0 is the fluorescence intensity at time zero (i.e., initial fluorescence of the vesicles), k_1 and k_2 are the rate constants for the first (fast) and second (slow) phases, respectively, and A_1 and A_2 are the amplitudes of the fast and slow phases, respectively. The fluorescence decays of liposomes are given by the single-exponential decay function $F(t) = F_0 - A \exp(-kt)$, where A and k are the amplitude and decay constant, respectively.

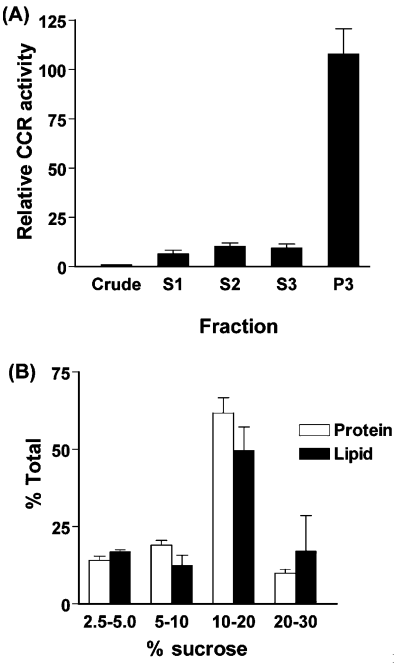

FIGURE 3: Isolation of spinach endoplasmic reticulum membranes and biochemical reconstitution of the detergent extract of salt-washed endoplasmic reticulum membranes to proteoliposomes. (A) Relative specific activity of cytochrome *c* reductase in different subcellular fractions: C, crude homogenate; S1, supernatant collected after centrifugation at 10000*g* for 15 min at 4 °C; S2, supernatant collected after S1 had been centrifuged at 40000*g* for 1 h at 4 °C; S3, supernatant collected after S2 had been centrifuged at 100000*g* for 1 h at 4 °C; P3, pellet collected after S2 had been centrifuged at 150000*g* for 2 h at 4 °C. The cytochrome *c* reductase assay was performed with a Perkin-Elmer spectrophotometer by monitoring the change in absorbance at 550 nm for 5 min at 25 °C. (B) Confirmation of reconstituted proteoliposomes by sucrose floatation gradient analysis. The *x*-axis shows the interfaces between two sucrose step gradients, and the *y*-axis shows the corresponding percent total lipid and amount of protein in each interface.

eral membrane proteins and centrifuged. The resulting pellet was further solubilized in buffer containing 1% (w/v) Triton X-100 to extract all the integral membrane proteins (that were supposed to contain flippases) and centrifuged as described in Experimental Procedures. The supernatant that was obtained (TE) was used as a source of flippases. A known quantity of Triton X-100-solubilized membrane fraction was added to ePC, and a trace quantity of either NBD-PC or NBD-PE (0.3 mol % of the ePC) was used to generate proteoliposomes by detergent removal using SM-2 Biobeads as described in ref *24*. Liposomes were prepared in similar fashion except that TE was not added. Maintaining a low bead/detergent ratio to promote the micellar to lamellar transition for the first 3 h of reconstitution has been described, and later the Biobead concentration can be increased to accelerate removal of detergent (Triton X-100) that might interact in subsequent flippase activity measurements. It has been shown that vesicles prepared in this manner are unilamellar with a mean diameter of ~150 nm, which was confirmed by size distribution analysis (*24*). Similarly, this method was followed to generate more or less uniform-sized (~100−150 nm) unilamellar vesicles during reconstitution of yeast ER microsomes (*19*). After reconstitution, vesicles were collected by centrifugation, washed to remove excess (non-reconstituted) fluorescently labeled analogues, and resuspended in assay buffer. To produce unilamellar and equal-sized vesicles, reconstituted vesicles were passed through a lipid extruder (11 times at least) using a 200 nm membrane. Scanning electron microscopy revealed that the mean size of vesicles was ~150 nm (data not shown). Protein and lipid recovery after reconstitution was found to be 50 and 60% (with respect to TE), respectively, which is in agreement with previously published reports (*15*).

To further confirm that TE proteins were reconstituted into vesicles, the reconstituted material was fractionated on a floatation sucrose gradient as described previously (*27*). The results clearly showed that ~80% of the protein and PL was recovered at the 20%−10% and 5%−10% (w/v) sucrose interfaces. However, proteoliposomes with a low PPR settled at a low percentage of sucrose (Figure 3B). The proteins that reconstitute into proteoliposomes float in the sucrose gradient along with lipid molecules, whereas the non-reconstituted proteins settle at the bottom of the centrifuge tube. The fraction of vesicles with a lower PPR (approximately <15% of the total protein and PL) settle at a lower sucrose density (2.5−5.0%), showing that a small fraction of vesicles has a lower PPR. These results strongly suggest that TE proteins were successfully reconstituted into proteoliposomes. Similar yields and recoveries were obtained when yeast microsomes were reconstituted into proteoliposomes (*19*).

Flippase Activity in Proteoliposomes Reconstituted with TE of the Spinach SWER. Liposomes and proteoliposomes generated from the Triton X-100 extract of the spinach SWER, ePC, and NBD-PC were treated with dithionite. In the case of liposomes (absence of flippase), a decrease in fluorescence to ~50% was observed due to quenching of NBD lipids on the outer leaflet. No further decrease in fluorescence was observed, since dithionite is impermeable to the membrane during the time course of the experiment (10−15 min) (Figure 4A,B). Upon addition of dithionite to proteoliposomes prepared with TE of spinach microsomal membranes, a sharp decrease in fluorescence to 50% of the initial fluorescence followed by a slow decrease in fluorescence was observed for both PC and PE (Figure 4A,B). This suggests that the sharp decrease in fluorescence was due to the quenching of labeled lipids on the outer leaflet, whereas the slower decrease in fluorescence was due to flipping of labeled PLs present in the inner leaflet to the outer leaflet and their subsequent quenching by dithionite. The percentage of quenching increased with the increase in protein concentration in reconstituted vesicles, and the percentage quenching also increased (Figure 4A,B), suggesting that flipping of NBD-PE and NBD-PC was protein-mediated. A linear relationship between the PPR and the amount of TE was observed when the protein concentration in the reconstitution mixture was increased to generate proteoliposomes with a higher PPR (Figure 4C).

Interestingly, the plot of percent NBD quenching versus PPR is different compared to the previously reported data (*16*). Percent NBD-PC and NBD-PE quenching increased linearly to a PPR of 40 mg/mmol and then attained a saturation value (Figure 4D). The saturation value can be interpreted as the vesicles in the assay mixture that contain at least one functional flippase. Vesicles reconstituted a a PPR between 0 and 40 mg/mmol possess zero or one functional flippase and thus fall in the linear region of flippase activity (*16, 29*). However, the saturation value (40 mg/

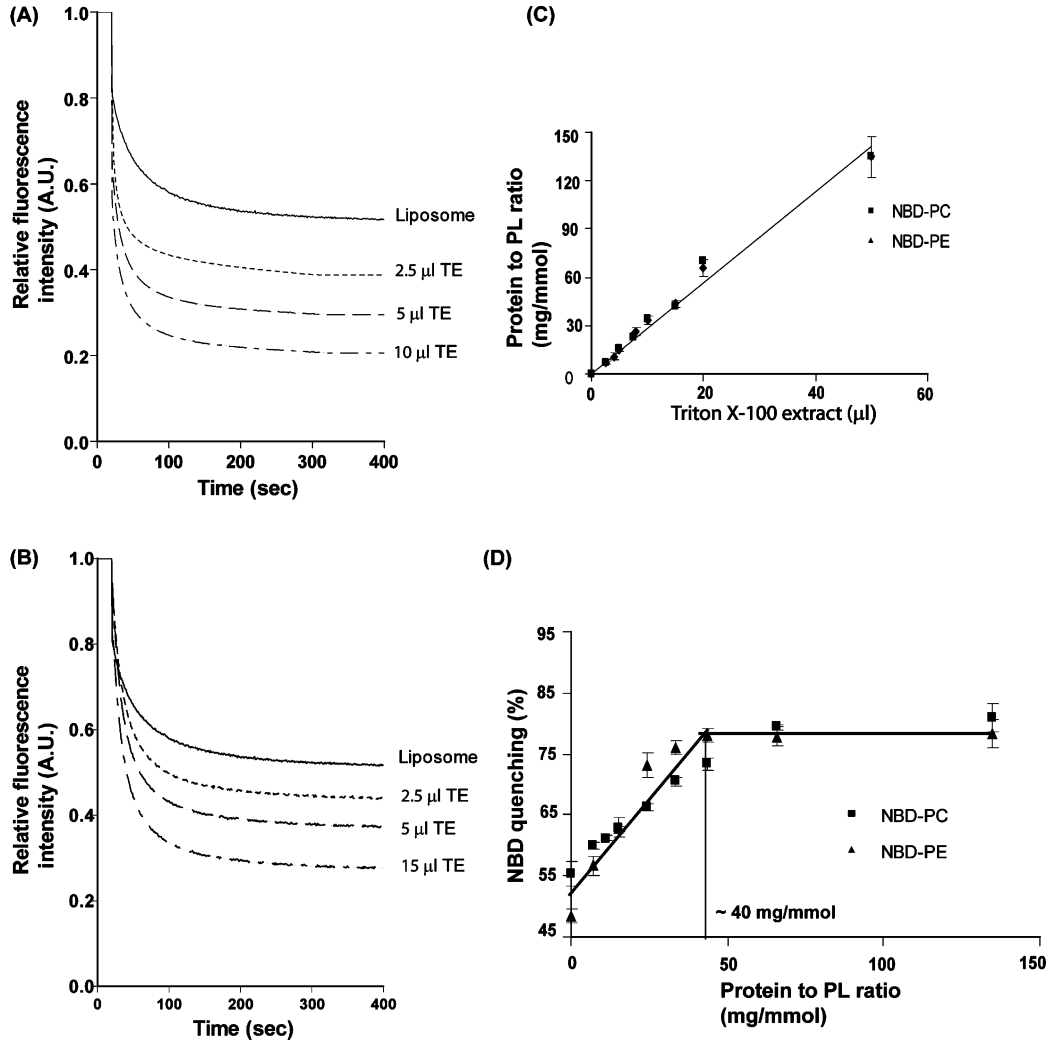

FIGURE 4: Flippase activity in proteoliposomes reconstituted from the detergent extract of spinach endoplasmic reticulum membranes. (A) The level of translocation of NBD-PC increases with an increase in the amount of protein in the proteoliposome. (B) The level of translocation of NBD-PE increases with an increase in the amount of protein in the proteoliposome. (C) Linear relationship between the amounts of TE to PPR in the reconstituted vesicles. (D) The extent of dithionite reduction of NBD-PC and NBD-PE depends on the PPR. Flippase activity increases proportionately with an increase in the PPR and reaches saturation at a PPR of 40 mg/mmol. Above this value, the percentage of fluorescence quenching remains unchanged for proteoliposomes.

mmol) obtained in this study was comparable to that obtained for rat liver microsomes (12 mg/mmol using NBD lipids as reporter molecules; 100 mg/mmol using ^{3}H-labeled dibutyrylPC), yeast microsomes (10 mg/mmol using NBD lipids as reporter molecules), and *Bacillus subtilis* cytoplasmic membranes (~40 mg/mmol) (*29, 14*). Since NBD-PC and NBD-PE reached a saturation value ~40 mg/mmol, it can be concluded that both are transported by a single class of flippase or by more than one class of flippase molecules with a similar abundance in spinach ER microsomal membranes. A careful analysis of the saturation point for flippase activities in various organisms shows that PPRs for rat liver and *Saccharomyces cerevisiae* microsomes are almost same (~10 mg/mmol), whereas the saturation PPRs for plants and bacteria are the same (~40 mg/mmol).

Kinetics of NBD-PC and NBD-PE Flipping in Proteoliposomes. The time courses of NBD-PC and NBD-PE flipping in proteoliposomes prepared from the Triton X-100 extract of the spinach SWER over time were fitted to a double-exponential decay function (Table 1). The first phase of the exponential decay is due to the rapid quenching of NBD-PC and NBD-PE on the outer leaflet. Irrespective of the PPR in proteoliposomes, it was found that the half-life of the first phase decay ($t^1_{1/2}$) was ~0.12 min (Table 1). This suggests that the first phase decay of fluorescence is not related to flipping and remains constant for the vesicles irrespective of the number of flippase molecules present in it. The second phase decay was much slower than the first phase ($t^2_{1/2}$ ~

Table 1: Kinetics of Translocation of NBD-PC and NBD-PE in Proteoliposomes[a]

sample	PPR (mg/mL)	$t^1_{1/2}$ (min)	$t^2_{1/2}$ (min)
liposome	0	0.12 ± 0.03	–
proteoliposome	2.5−40.0	0.126 ± 0. 04	1.02 ± 0.18
proteoliposome	40.0−80.0	0.130 ± 0.001	0.8 ± 0.011
proteoliposome	80.0−140.0	0.113 ± 0.007	0.72 ± 0.022

[a] $t^1_{1/2}$ is the half-life of the exponential decay of the fluorescence present in the outer leaflet. $t^2_{1/2}$ is the half-life of the translocation of PLs from the inner leaflet to the outer leaflet, thereby becoming quenched with dithionite. The calculation of half-life was performed by using the equation $F(t) = F_0 - [A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t)]$, where $F(t)$ is the fluorescence as a function of time, F_0 is the fluorescence intensity at time zero (i.e., initial fluorescence of the vesicles), k_1 and k_2 are the rate constants for the first (fast) and second (slow) phases, respectively, and A_1 and A_2 are the amplitudes of the fast and slow phases, respectively.

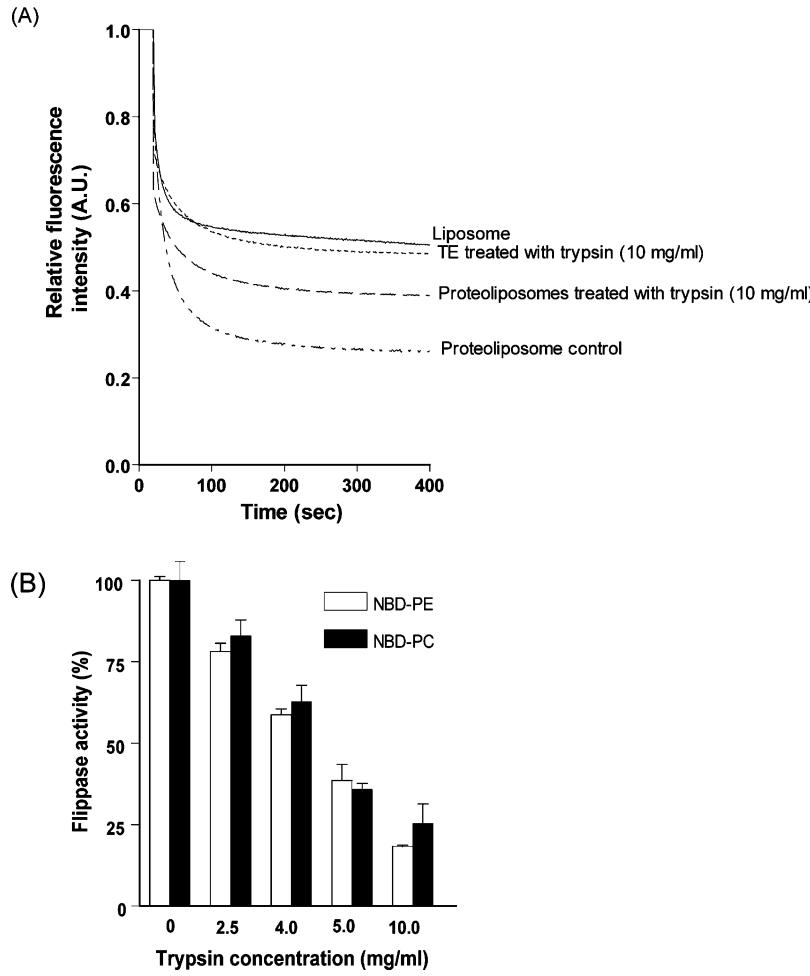

FIGURE 5: Effect of protease (trypsin) on the flippase activity of spinach ER membrane proteins. (A) Both liposomes and proteoliposomes (PPR ~ 30 mg/mmol) were treated with trypsin at 10 mg/mL and incubated for 30 min at 37 °C. TE was treated with same amount of trypsin under similar conditions prior to reconstitution into proteoliposomes. (B) Dose-dependent decrease in the flippase activity of spinach ER membrane proteins reconstituted into proteoliposomes upon trypsin treatment. Liposomes (50 μL) were incubated with the desired concentration of bovine pancreatic trypsin at 37 °C for 30 min. The assay was performed in a Jasco spectrofluorimeter for 10 min at 25 °C.

0.7−1.0 min), which corresponds to the protein-mediated flipping of NBD-PCs from the inner leaflet to the outer leaflet and their subsequent quenching by dithionite (Table 1). A similar rate of translocation was also observed for NBD-PE. The half-time of flipping was found to be 0.5 min in yeast microsomes for NBD-PC (*29*), 1−3 min in *E. coli* cytoplasmic membranes for NBD-PC and NBD-PE (*12*), 0.5 min in *Bacillus megaterium* vesicles for NBD-PC and NBD-PE (*14*), and 3 min in liver ER and Golgi membranes for monohexosyl sphingolipid phospholipids (*11*). Our data for the half-life of flipping from spinach ER match the rate of flipping measured in yeast, bacteria, and mammals.

Interestingly, we also found that the second phase half-life was more or less constant (~1 min) for vesicles prepared with a PPR of 0−40 mg/mmol (Table 1). Beyond this ratio (40−80 mg/mmol), the second phase half-life was reduced to 0.8 min, suggesting that the rate of flipping was increased. This can be interpreted as showing that the increase in the PPR above 40 mg/mmol results in vesicles with more than one flippase per vesicle on average (*15*). Hence, no change in the amplitude of NBD quenching was observed (see Figure 4D) beyond 40 mg/mmol, but an increase in the rate of translocation or a decrease in the second phase half-life was observed.

Effect of Protease on Flippase Activity in Reconstituted Proteoliposomes. To further confirm that flipping of PC and PE was protein-mediated, we treated proteoliposomes (PPR ~ 30 mg/mmol) with trypsin for 20 min at 37 °C and assayed for flippase activity (Figure 5A). For a control, liposomes were treated with trypsin under identical conditions and proteoliposomes were also mock treated. Figure 5A shows that protease treatment reduced the level of NBD quenching, which in turn shows that flipping activity was reduced. This suggests that trypsin treatment resulted in a reduction in the number of flippase active vesicles. Dose-dependent protease treatment showed a proportionate decrease in the level of NBD quenching with an increase in trypsin concentration. Among the reporter molecules, flipping of NBD-PE was more affected (~82 ± 4% at 10 mg/mL trypsin) than that of NBD-PC (~75 ± 2% at 10 mg/mL) only after protease treatment (Figure 5B). Alternatively, we also treated the TE with 10 mg/mL trypsin prior to reconstitution as described above. Reconstitution of trypsin-treated TE into vesicles showed ~50% PFR, which was similar to that of liposomes (Figure 6A). These results strongly suggest that (i) flipping of PC and PE in spinach membranes is protein-mediated, (ii) flippase activity can be completely abolished when TE is treated with trypsin prior to reconstitution, (iii) trypsin

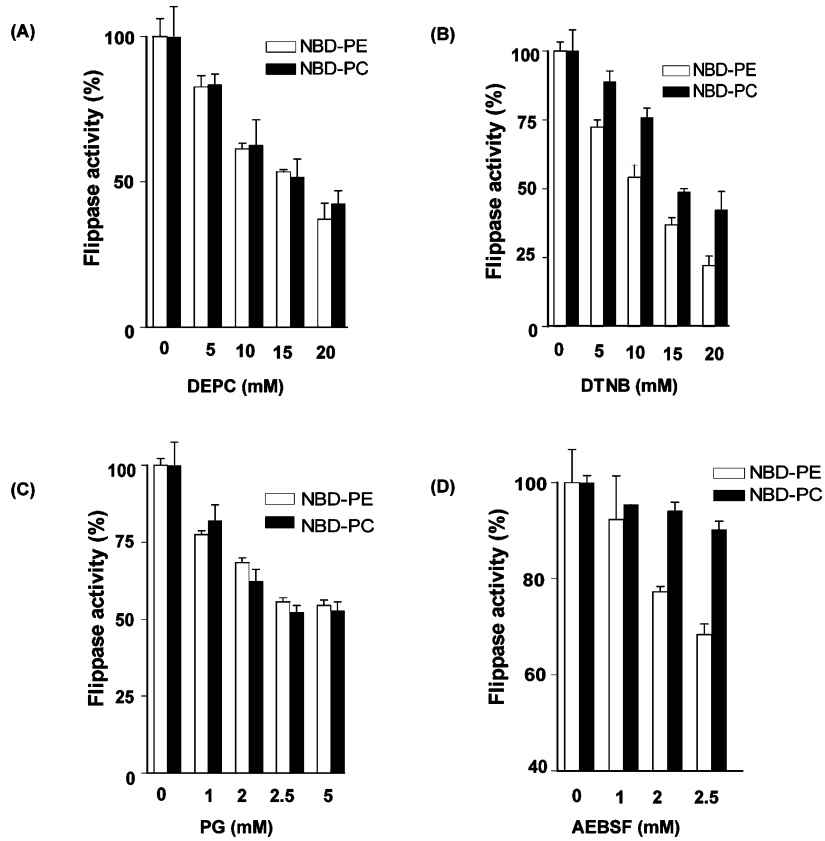

FIGURE 6: Effect of protein-modifying reagents on the flippase activity of proteoliposomes reconstituted from *S. oleracea* L. ER membranes. Proteoliposomes were incubated with the desired concentration of protein-modifying reagents at 37 °C for 30 min. (A) Effect of DEPC on flippase activity. (B) Effect of DTNB on flippase activity. (C) Effect of PG on flippase activity. (D) Effect of AEBSF on flippase activity.

treatment after reconstitution could not abolish flippase activity completely, implying that some portion of flippase was protected inside the membrane bilayer upon reconstitution, and (iv) spinach flippases are more sensitive to protease than rat liver flippases (*16*). Due to the difference in inhibition of NBD-PC- and NBD-PE-mediated translocation upon protease treatment, we hypothesize that more than one functionally distinct protein or class of proteins is present in spinach membranes.

Effect of Protein-Modifying Reagents on Flippase Activity. To further confirm this hypothesis, we checked the sensitivity of flipping of both NBD-PE and NBD-PC to chemical modifications with various protein-modifying reagents such as diethyl pyrocarbonate (DEPC) for histidine, phenyl glyoxal (PG) for arginine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) for cysteine, and 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) for serine (*30*−*33*). Proteoliposomes (PPR ∼ 30 mg/mmol) were treated with protein-modifying reagents at different concentrations for 15 min at 25 °C and assayed for flippase activity (Figure 6). Flippase activity was more affected when proteoliposomes were modified with cysteine followed by histidine, arginine, and serine. Interestingly, no difference in PC and PE flipping was observed when proteoliposomes were treated with different concentrations of DEPC and PG (Figure 6A,C). However, remarkable differences between PC and PE flipping were observed when they were treated with DTNB and AEBSF (Figure 6B,D). Earlier experiments with rat liver flippase showed that *N*-ethylmaleimide (NEM), an analogue of DTNB, reduced flippase activity by ∼40% and had identical effects on NBD-PC and NBD-PE (*16*). In contrast, inhibition of PC flipping and inhibition of PE flipping are not identical, and a greater level of inhibition (78 ± 3.4% for PE and 58 ± 6.4% for PC) was observed for spinach flippases only after treatment with DTNB or AEBSF. No further improvement in inhibition was observed beyond the concentrations of modifying reagents used in this study.

To gain insight into the differential modification of NBD-PC- and NBD-PE-mediated flippase activity, we treated the TE with DTNB (20.0 mM) and AEBSF (5.0 mM) prior to reconstitution and incubated the mixture at 25 °C for 30 min. This pretreated TE was reconstituted into proteoliposomes (PPR ∼ 30 mg/mL); flippase activity was measured, and the same amount of TE was mock treated and reconstituted for the positive control (Figure 7A,B).

The inhibition of flippase activity for PC (∼60%) and PE (∼80%) following DTNB treatment was almost similar for TE prior to reconstitution and TE after reconstitution into proteoliposomes (Figure 7A). However, a significant difference in inhibition was observed between proteoliposomes reconstituted from AEBSF-treated TE (treatment prior to reconstitution) (∼75% for PC and ∼72% for PE) and AEBSF-treated proteoliposomes (treatment after reconstitution) (∼10% for PC and 32% for PE) (Figure 7B). It should also be noted that there was no significant difference in flipping for NBD-PC and NBD-PE for AEBSF-treated TE prior to reconstitution. However, a significant difference in flipping was observed for PC and PE when TE was treated with the other agent, DTNB, prior to reconstitution (Figure 7A,B). On the basis of the sensitivity to DTNB and AEBSF

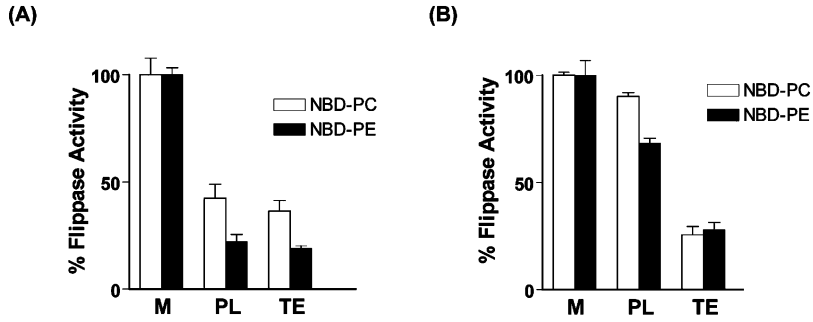

FIGURE 7: Effect of (A) DTNB (20 mM) and (B) AEBSF (5.0 mM) on flippase activity when TE was treated prior to reconstitution: M, mock (untreated) proteoliposomes; PL, proteoliposomes treated with a protein-modifying reagent (20 mM DTNB or 5 mM AEBSF); TE, Triton X-100 extract treated with a protein-modifying reagent (20 mM DTNB or 5 mM AEBSF) prior to reconstitution. Proteoliposomes were incubated with the desired concentration of protein-modifying reagents at 37 °C for 30 min.

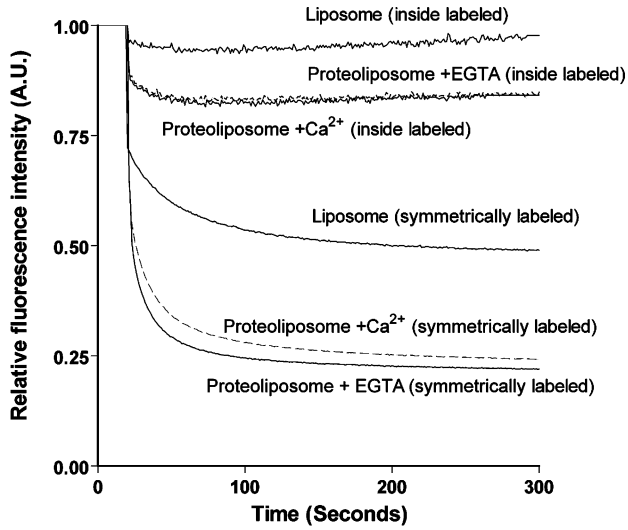

FIGURE 8: Scramblase assay for symmetrically labeled and inside-labeled proteoliposomes generated from the detergent extract of salt-washed spinat endoplasmic reticulum membranes. Both the EGTA-treated (4.0 mM) and Ca^{2+}-treated (2.0 mM) vesicles exhibited the same level of quenching of NBD-PLs in either inside-labeled or symmetrically labeled vesicles, showing the absence of scramblase activity.

and consequent differences in inhibition of PC and PE flipping, we can interpret that more than one class of flippases exists in spinach ER.

Is Flipping Activity Due to Scramblase? To test if the flippase activity present in the ER membrane is a scramblase (calcium-mediated rapid scrambling of PLs)-mediated phenomenon, we tested for scramblase activity in vesicles containing spinach ER membrane protein (PPR $\sim$ 30 mg/mmol) in both inside-labeled and symmetrically labeled vesicles. Vesicles were dialyzed several times to remove calcium, and after dialysis, 4 mM EGTA was added to make sure that no free calcium was available during activity measurements. Figure 8 shows that there was no calcium-dependent increase in the level of quenching of NBD-PLs for proteoliposomes (both symmetrically labeled and inside-labeled) reconstituted from spinach ER membranes. These results show that the protein-dependent flippase activity was not due to scramblase. Our data are consistent with the previous findings in that the scramblase is not present in ER and whatever activity is obtained is due to only biogenic membrane flippase (*21*).

CONCLUSIONS

In this study, we present the first evidence of ATP-independent PL translocation in proteoliposomes reconstituted from the Triton X-100 extract of spinach ER microsomes. Trypsin treatment and dose-dependent addition of TE to reconstituted vesicles confirmed that PC and PE flipping is protein-mediated. Saturation of flippase activity for PC and PE at 40 mg/mmol suggests that both are translocated by a single class or more than one class of flippases with similar abundance. Results indicate that the abundance of flippase in the spinach ER is lower than that in the rat liver ER but is similar in comparison with that in bacterial membranes. In an earlier study of rat liver ER flippase, it has been shown that PC, PE, and PS flipping was inhibited equally when membranes were treated with NEM and DEPC (*16*). In contrast, spinach flippase exhibited a different degree of inhibition for PC and PE when treated with DTNB and AEBSF. These results strongly support our hypothesis that more than one class of lipid translocator is present in spinach membranes, which is differentially modified with reagents and affects translocation of PC and PE differently.

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