Biochimica et Biophysica Acta 862 (1986) 343–351
Elsevier

BBA 73342

Phosphatidylserine decarboxylase: generation of asymmetric vesicles and determination of the transbilayer distribution of fluorescent phosphatidylserine in model membrane systems

Yvonne M. Denkins and Alan J. Schroit

Department of Cell Biology, The University of Texas, M.D. Anderson Hospital and Tumor Institute at Houston, Houston, TX 77030 (U.S.A.)

(Received 27 June 1986)

Key words: Phosphatidylserine; Phosphatidylserine decarboxylase; Fluorescent lipid; Membrane asymmetry

Large unilamellar vesicles (LUV) that contained a fluorescent analog of phosphatidylserine (NBD-PS) were used in model systems to determine the feasibility of employing phosphatidylserine decarboxylase (PS-decarboxylase) to generate asymmetric vesicles and to determine the transbilayer distribution of PS. PS-decarboxylase prepared by sonication of *Escherichia coli* JA 200 pLC 8-47 was found to be stable in detergent-free buffers and catalyzed the conversion of NBD-PS to NBD-phosphatidylethanolamine (NBD-PE). PS-decarboxylase was capable of decarboxylating virtually all of the NBD-PS present in the outer leaflet of LUV containing a symmetric or asymmetric (outside only) distribution of NBD-PS, but not NBD-PS present in the inner leaflet of the vesicles. The ability of PS-decarboxylase to decarboxylate only NBD-PS located in the outer leaflet of the vesicles was independently verified by resonance energy transfer (between NBD-PS and (lissamine) rhodamine B-labeled phosphatidylethanolamine) and by derivatization with trinitrobenzenesulfonic acid (TNBS). These techniques revealed that the exchangeable pool (the fraction of NBD-PS on the outer leaflet) and the respective fraction of Tnp-(NBD-PS) formed were equivalent to the extent of PS-decarboxylase-mediated decarboxylation of NBD-PS to NBD-PE. These results show that PS-decarboxylase can be used to generate asymmetric vesicles (i.e., PS inside, PE outside) and determine the intrabilayer distribution of PS in model membranes.

Introduction

The use of chemical labeling agents and phospholipases have shown that anionic phospholipids such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) are asymmetrically distributed in biological membranes [1,2]. In red blood cells (RBC) phosphatidylcholine is mainly localized in the outer leaflet while the amino lipids, in particular phosphatidylserine (PS), are preferentially distributed in the inner cytoplasmic face [3,4].

While the asymmetric distribution of lipids in normal erythrocytes can be perturbed by a variety

Abbreviations: ^{125}I-PE, *N*-[3-(3-[^{125}I]iodo-4-hydroxybenzyl)-propionyl]dipalmitoylphosphatidylethanolamine; DOPC, dioleoylphosphatidylcholine; Rho-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; NBD-PC, -PS, -PE, -COOH, -PA, 1-acyl-2-[(*N*-4-nitrobenz-2-oxa-1,3-diazole)-aminocaproyl]phosphatidylcholine, -serine, -ethanolamine, -*N*-succinylethanolamine; -phosphatidic acid; PBS, Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (pH 7.4); PSdC, phosphatidylserine decarboxylase; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid; Tnp, trinitrophenyl; LUV, large unilamellar vesicles.

Correspondence: Dr. A.J. Schroit, Department of Cell Biology, The University of Texas, 6723 Bertner Avenue, Houston, TX 77030, U.S.A.

of agents [5–7], the "spontaneous" translocation of lipids from the inner to outer monolayer has been implicated in the process of homeostasis. For example, the translocation of PS in activated platelets [8] has been shown to be important in the prothrombin converting activity of these cells [9], and in sickled erythrocytes [10,11] PS translocation has been implicated as a possible factor in the pathogenesis of sickle cell anemia [12,13]. Since the rate of phospholipid movement across biological membranes is rapid compared to synthetic membrane systems the existence of specific proteins have been suggested to be responsible for the rapid transbilayer movement in cell membranes [14]. The possible existence of lipid-specific protein transporters [15,16] that control lipid movement and maintain a particular transbilayer distribution of lipids is intriguing, especially in the case of PS due to the pathological consequences of its exposure in the outer leaflet of erythrocytes [12,13]. The search for such proteins, however, has been impeded by the lack of efficient techniques for the generation of asymmetric PS vesicles and an analytical method for determining the intrabilayer distribution of PS.

Here, we present a new method that employs phosphatidylserine decarboxylase (PS-decarboxylase) [17–19], which catalyzes the conversion of PS to PE, as a tool to generate asymmetric vesicles, and as an analytical method for determining the transbilayer distribution of PS in artificially generated model membrane systems. We show that PS-decarboxylase is capable of decarboxylating all of the NBD-PS present in the outer leaflet of large unilamellar vesicles (LUV) containing uniformly distributed NBD-PS, thereby producing vesicles with an asymmetric lipid distribution (PS localized only in the inner leaflet).

Materials and Methods

Methods and routine procedures

PS (bovine brain), DOPC, Rho-PE and NBD-PC were purchased from Avanti Polar lipids (Birmingham, AL), phospholipase D (cabbage) from Boehringer-Mannheim, and phospholipase C (*Clostridium perfringens*) from Calbiochem-Behring. JA 200 pLC 8-47, an *Escherichia coli* strain bearing the hybrid plasmid COlE1-psd$^+$ [20] for the over-production of PS-decarboxylase [21] was obtained from Dr. Barbara Bachmann, Yale University Gene Bank, New Haven, CT. All lipids were stored at −70°C and monitored for purity by TLC using activated silica gel 60 thin-layer plates (Merck). Lipid concentrations were determined according to Ames and Dubin [22] or Fiske and SubbaRow [23]. Protein was determined as described by Lowry et al. [24].

Preparation of PS-decarboxylase and determination of its activity

PS-decarboxylase from *E. coli* strain JA 200 pLC 8-47 was prepared from stock *E. coli* grown to late log phase in Bacto tryptone medium. After washing, the cells were resuspended in 0.1 M phosphate buffer (pH 7.0) containing 10% glycerol. The cells were then sonicated at 4°C, using a Heat Systems model W-375 probe sonifier at 50 watts (three 1 min bursts with intermittent cooling). The sonicate was then centrifuged at 20000 × g for 30 min, and the supernatant was collected and frozen. Enzymatic activity was assessed by its ability to decarboxylate NBD-PS and bovine brain PS. Briefly, aliquots of the enzyme were added to a suspension of PS vesicles in the presence or absence of Triton X-100 (0.1% final concentration). The suspension was then incubated at 37°C and terminated at various intervals by acidification with 0.1 M HCl. The reaction mixture was then partitioned by the addition of $CHCl_3$ and methanol and the fraction of PE formed (decarboxylated PS) was determined after its separation from the residual substrate (PS) by TLC using $CHCl_3/CH_3OH/28\%$ NH_4OH (65 : 35 : 5, v/v) as the solvent system. The specific activity of PS-decarboxylase using this procedure was found to be approx. 300 units/mg protein in the absence of Triton X-100 and approx. 150 units/mg in the presence of Triton X-100 for NBD-PS and bovine PS, respectively (one unit = nmol PS decarboxylated per min at 37°C). Phosphate analysis showed that the PS-decarboxylase contained 60 nmol lipid phosphate (> 90% PE)/mg protein.

Fluorescent lipids

NBD-PS and NBD-PE were prepared by phospholipase D-catalyzed base exchange of NBD-PC in the presence of L-serine and ethanolamine, re-

spectively [25]. NBD-PA was isolated as a byproduct from the NBD-PC/ethanolamine reaction mixture. NBD-COOH (succinylated NBD-PE) was prepared from NBD-PE by condensation with succinic anhyride [26] and ^{125}I-labeled PE (^{125}I-PE) was synthesized as described previously [27]. Analysis of the purified phospholipid products revealed single fluorescent-, ninhydrin- and/or phosphate-positive spots. The amino-containing lipids were positively identified on the basis of their reactivity with TNBS by assessing their altered mobility on TLC plates.

Vesicle preparation

Vesicles were prepared from appropriate lipid mixtures after the solvents were removed by evaporation under nitrogen and high vacuum for at least 1 h. LUV were prepared by ethanol injection [28]. Small unilamellar acceptor vesicles (SUV) were prepared by ultrasonication (17 mg of DOPC/ml) or by ethanol injection [28]. All ethanol containing vesicles were dialyzed overnight against phosphate-buffered saline (PBS) at 4°C.

Generation of asymmetric vesicles

LUV containing a preferential distribution of NBD-PS in the inner of outer leaflet were prepared as follows. NBD-PS in the inner leaflet: LUV containing a symmetric distribution of NBD-PS were prepared from DOPC/NBD-PS/Rho-PE (98 : 1 : 1, molar ratio) as described above. These 'donor' LUV were then mixed with a 5-fold excess (w/w) of 'acceptor' SUV (prepared by ultrasonication of DOPC with trace amounts of ^{125}I-PE and pre-sized on Bio-Gel A-15M) and incubated overnight at 20°C. The mixed vesicle populations were then separated by chromatography on Bio-Gel A-15M (1.1 × 45 cm). Fractions were collected and monitored by fluorescence and scintillation counting. The excluded peak was then adjusted to an appropriate lipid concentration based on rhodamine fluorescence. NBD-PS in the outer leaflet: LUV were prepared from DOPC/Rho-PE (99 : 1) as described above. The LUV (1.7 mg of lipid) were mixed with 15 μg of NBD-PS that had been dried on the walls of a small glass tube. After several minutes at 20°C the mixture was chromatographed on a Bio-Gel A-15M column (1.1 × 45 cm). Column fractions were sequentially analyzed for energy transfer efficiency (λ_{ex} 450 nm; λ_{em} 530 nm) and light scatter ($A_{320\ nm}$). The amount of NBD-PS incorporated into the LUV was determined by quantifying the extent of NBD fluorescence in the presence of detergent and comparing it to a standard curve generated from known amounts of NBD-PS. The LUV were then adjusted to an appropriate concentration based on rhodamine fluorescence.

Fluorescence measurements

Steady-state fluorescence was quantified at 530 nm (λ_{ex} 468 nm) with a dual-beam Farrand MKII spectrophotofluorometer at 20°C using 2.5 nm excitation and emission slits and an automated TLC plate scanner. Data acquisition was controlled by an Apple IIe computer that sampled and digitized data from the fluorometer at 0.5 s/1.6 mm intervals. The linearity of fluorescence obtained from the plate scanner was verified by performing standard curve calibrations on chromatographed samples (reading directly from the plate), and it was found to be as linear (> 0.9) as the values obtained from conventional fluorescence readings using the same standards in cuvettes.

Resonance energy transfer assay

The resonance energy transfer assay for measuring the transfer of NBD-labeled lipids between two vesicle populations was done essentially as described by Pagano et al. [29] using exchangeable NBD-PS as the energy donor and nonexchangeable Rho-PE [30] as the energy acceptor. Briefly, the energy transfer efficiency of the initial donor vesicle population was determined by measuring the fluorescence at 530 nm (λ_{ex} 468 nm) of a 15 μl aliquot of the vesicles (DOPC/NBD-PS/Rho-PE, 98 : 1 : 1) in 1.3 ml phosphate-buffered saline before and after the addition of Triton X-100 (1.25% final concentration). Typically, values of 90–95% were obtained. The amount of totally exchangeable lipid (assumed to be lipid residing in the outer leaflet) was determined by adding 1.0 ml of unlabeled acceptor vesicles to 15 μl of donor vesicles (donor/acceptor lipid ratio, 1 : 100) in 300 μl of phosphate-buffered saline, using the same concentration of acceptors alone in the reference

cuvette. The total exchangeable fluorescence lipid pool was determined after the fluorescence reached equilibrium. The amount of NBD-lipid/relative fluorescence intensity was determined after the addition of Triton X-100 (1.25% v/v final concentration) and compared to a standard curve generated from known amounts of standard NBD-lipid in exactly the same amount of total non-fluorescent lipid at the same temperature. The fluorescent yield of the NBD-lipid in the Triton X-100 lysates was corrected for dilution and for detergent-mediated quenching [31] and was found to be approx. 60% of the fluorescence measured in intact (nonquenched) vesicles. The fraction of total fluorescent lipid transferred (at equilibrium) was calculated using the following equation:

$$\text{Fraction (lipid in outer leaflet)} = (F_{eq} - F_i)/(F_t - F_i)$$

where F_{eq} is the relative fluorescence of the donor vesicle population at equilibrium with excess acceptors; F_t is the total amount of fluorescence found after the addition of Triton X-100 (corrected for detergent-mediated quenching and dilution, see above); and F_i is the fluorescence of the initial donor vesicle population in the absence of acceptor vesicles.

Trinitrophenylation of vesicles

An alternative method for determining the fraction of aminophospholipids residing in the outer LUV leaflet was derivatization with TNBS under nonpermeable conditions [3,32]. Briefly, vesicles were trinitrophenylated with 1.5 mM TNBS at pH 8.5 using an isotonic bicarbonate buffer (120 mM sodium bicarbonate; 40 mM NaCl) for 30 to 45 min at 2°C. Preliminary trials revealed that the reaction plateaued during this period, resulting in approx. 50% derivatization of the aminophospholipids. The reaction was stopped by the addition of 0.1 M HCl and extracted with $CHCl_3/CH_3OH$. The fraction of derivatized PS (Tnp-PS) in the organic phase was quantified by appropriate techniques (i.e., P_i assay, fluorescence) after its isolation by TLC.

Results

Decarboxylation of NBD-PS

Since it has recently been shown that PS-decarboxylase activity may be dependent on the acyl-chain composition of the substrate and on the presence of detergent [19,33], initial experiments were carried out on sonicated lipid dispersions of NBD-PS in the presence and absence of Triton X-100, using NBD-PC, NBD-PE, NBD-PA and NBD-COOH as controls. The reactions were stopped after 1 h incubation at 37°C by the addition of 0.1 M HCl and extracted and analyzed by TLC. The results shown in Fig. 1 indicate that the preparation decarboxylated NBD-PS irrespective of the presence of detergent, and that it did not contain other lipases since no alterations in the relative mobilities of the other NBD analogs could be detected.

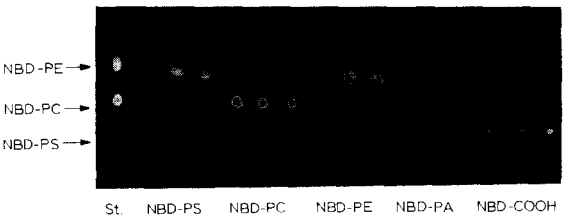

Fig. 1. Thin-layer chromatogram of PS-decarboxylase-treated NBD-phospholipid analogs. 5 μg of the indicated fluorescent phospholipid analogs were dispersed in 250 μl phosphate-buffered saline and exposed to PS-decarboxylase (50 μg) in the presence or absence of Triton X-100 (1.25% final concentration) for 1 h at 37°C. The reactions were stopped by the addition of 0.1 M HCl, extracted and chromatographed. Within groups, the lanes represent NBD-lipid alone (left); NBD-lipid with PS-decarboxylase (center); and NBD-lipid with PS-decarboxylase and Triton X-100 (right). Far left lane contains lipid standards.

Decarboxylation of NBD-PS in LUV

To assess the ability of PS-decarboxylase to decarboxylate NBD-PS in a bilayer membrane, LUV were formed from DOPC/NBD-PS (99 : 1), mixed with PS-decarboxylase, and incubated at 37°C. At appropriate time intervals, aliquots were removed, extracted, and analyzed by quantitative TLC. The data presented in Fig. 2 indicate that the decarboxylation of NBD-PS to NBD-PE in LUV is time dependent, with maximum conversion (approx. 50%) occurring during the first 60 min of incubation. Interestingly, increasing the incubation time up to 24 h did not result in increased production of NBD-PE.

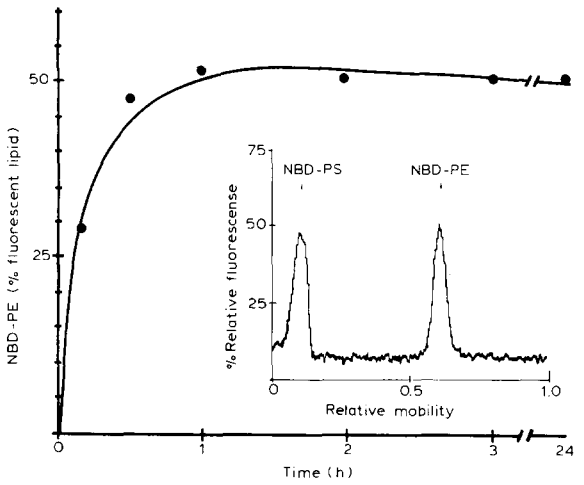

Fig. 2. Decarboxylation of NBD-PS in LUV. LUV composed of DOPC/NBD-PS (99:1) were mixed with PS-decarboxylase (185 μg lipid/143 μg PS-decarboxylase per ml) at 37°C. At the indicated time intervals aliquots were removed, extracted and chromatographed. The relative NBD fluorescence (λ_{ex} 468 nm; λ_{em} 530 nm) of the individually scanned lanes was quantified by integration. Inset: Typical TLC scan.

The inability to decarboxylate more than about half of the NBD-PS was apparently not caused by significant losses of enzymatic activity, since incubation of PS-decarboxylase alone for 20 h, and the subsequent addition of LUV for 1 h, resulted in only approx. 37% reduction in the extent of NBD-PS decarboxylation. Thus, fresh enzyme decarboxylated 52% of the NBD-PS in LUV, whereas PS-decarboxylase pre-incubated at 37°C for 20 h hydrolyzed 33% of the NBD-PS.

These results demonstrate that PS-decarboxylase can decarboxylate about half of the NBD-PS present in the vesicles. These observations might indicate that the enzyme is capable of decarboxylating only NBD-PS present in the vesicles' outer leaflet thereby producing preparations with an asymmetric distribution of PS. In an attempt to verify this possibility, we investigated whether PS-decarboxylase could decarboxylate NBD-PS to NBD-PE in asymmetric vesicles generated by lipid transfer techniques [29].

Quantitative determination of NBD-PS asymmetry in LUV

To determine whether PS-decarboxylase decarboxylated only NBD-PS present in the outer leaflet of LUV, vesicles were prepared that contained NBD-PS in (a) only the inner leaflet, (b) only the outer leaflet, or (c) in both leaflets. Thus, when DOPC/NBD-PS/Rho-PE (98:1:1) donor LUV were allowed to equilibrate with excess

TABLE I

PREPARATION OF ASYMMETRIC LUV

Initial vesicle composition	Acceptor/donor composition	Pre-column vesicle composition			Post-column vesicle composition [c]		
		Rho [d] (μg)	NBD [e] (μg)	RET [f] (%)	Rho [d] (μg)	NBD [e] (μg)	RET [f] (%)
Inner leaflet label [a]							
DOPC/Rho-PE/NBD-PS	DOPC/^{125}I-PE	18.8	18.8	85	15	9.6	78
Outer leaflet label [b]							
DOPC/Rho-PE	NBD-PS	18.8	0.0	–	14.4	9.7	86

[a] Inner leaflet NBD-PS-labeled vesicles were formed by incubating DOPC/Rho-PE/NBD-PS (98:1:1) LUV with a 5-fold excess (w/w) of a homogeneous SUV (DOPC/^{125}I-PE) acceptor population for 20 h at 20°C.

[b] Outer leaflet NBD-PS-labeled vesicles were formed by incubating DOPC/Rho-PE (99:1) LUV with 15 μg of NBD-PS (see Materials and Methods) for 2 min at 20°C.

[c] LUV-SUV mixtures were separated by column chromatography on a Bio-Gel A-15M (1.1×45 cm).

[d] Relative fluorescence (λ_{ex} 560 nm; λ_{em} 600 nm) of nonexchangeable Rho-PE served as an internal standard for vesicle weight, i.e. Rho-PE is 1% of the total DOPC carrier.

[e] NBD-PS was quantified in the presence of Triton X-100 (λ_{ex} 468 nm; λ_{em} 530 nm) using an appropriate standard curve generated from known amounts of NBD-PS in the same solvent.

[f] The resonance energy transfer efficiency (RET) of the LUV populations was calculated using $1 - F_i/F_t$ (see Experimental Procedures).

acceptor DOPC/^{125}I-PE (100 : trace) SUV, approx. 40% of the NBD-PS (representing the fraction of lipid localized in the LUV outer leaflet) transferred to the SUV acceptors, albeit with only small alterations in the resonance energy transfer efficiency of the isolated LUV population (Table I). Furthermore, most of the LUV were recovered from the column (based on nonexchangeable Rho-PE fluorescence) with no significant contamination (< 0.2%) by the ^{125}I-labeled acceptor population. Conversely, when the LUV were employed as lipid acceptors (DOPC/Rho-PE, 99 : 1), most of the NBD-PS transferred to the vesicles resulting in the concomitant development of resonance energy transfer (Table I).

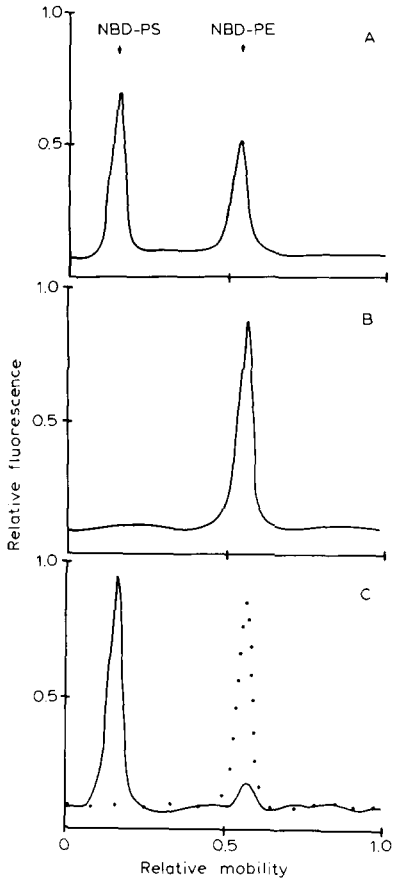

Fig. 3. Decarboxylation of NBD-PS in asymmetric vesicles. Asymmetric and symmetric vesicles (prepared as described in Table I) were incubated with PS-decarboxylase in the presence or absence of Triton X-100 as described for Fig. 2. The reactions were stopped by the addition of 0.1 M HCl and the lipids extracted and chromatographed. The plates were then scanned and the peak areas quantified. (a) symmetric LUV; (b) asymmetric LUV, NBD-PS outside; (c) asymmetric LUV, NBD-PS inside. ———, vesicles incubated in the absence of detergent; ●, vesicles incubated in the presence of detergent.

The degree of NBD-PS asymmetry in these artificially generated LUV was determined using PS-decarboxylase, and verified independently on the same preparation by resonance energy transfer assay [29] and by derivatization with TNBS [3,32]. The ability of PS-decarboxylase to decarboxylate NBD-PS in these vesicles is shown in Fig. 3. It can be seen that PS-decarboxylase decarboxylated approx. 35% of the NBD-PS associated with the symmetric vesicles (Fig. 3A), 97% of the NBD-PS in the asymmetric outer leaflet-labeled vesicles (Fig. 3B), and only approx. 5% (possibly representing incomplete removal from the outer leaflet) of the NBD-PS present in the inner leaflet-labeled vesicles (Fig. 3C). Fig. 4 shows the size of the exchangeable pool, an indication of the fraction of NBD-PS in the outer leaflet, as determined after equilibration with an excess of unlabeled acceptors. In the initial 'symmetric' vesicle population, approx. 40% of the NBD-PS transferred out of the donor population, whereas virtually all or none of the NBD-PS transferred out of asymmetric 'outside-only' NBD-PS and 'inside-only' NBD-PS

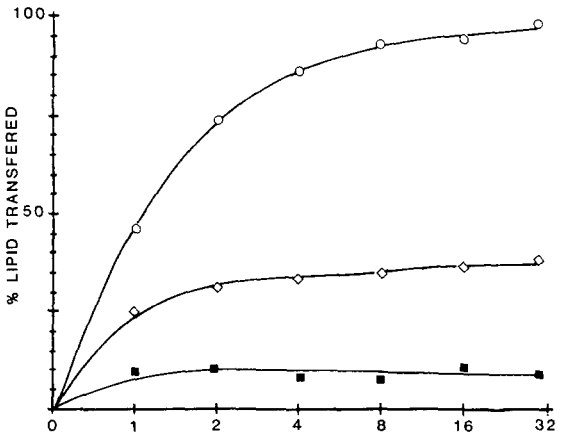

Fig. 4. Exchangeable NBD-PS pool in artificially generated asymmetric vesicles. Asymmetric donor vesicles (see Table I legend) were incubated for 20 h at room temperature with increasing amounts of small unilamellar DOPC acceptor vesicles. The resonance energy transfer efficiency of the mixed vesicle population was then assessed (see Materials and Methods). ◇, symmetrically distributed NBD-PS; ■, NBD-PS in the inner leaflet; ○, NBD-PS in the outer leaflet.

TABLE II

DETERMINATION OF NBD-PS DISTRIBUTION IN ASYMMETRIC LUV [a]

Vesicle type	Fraction of outer leaflet NBD-PS determined by		
	PSdC [b]	Energy transfer [c]	TNBS [d]
Uniform NBD-PS	0.36	0.34	0.35
Outer leaflet NBD-PS	0.97	0.99	0.99
Inner leaflet NBD-PS	0.08	0.08	0.50

[a] Asymmetric LUV were prepared and purified as described in Table I.

[b] The fraction of outer leaflet NBD-PS = NBD-PE/(NBD-PE + NBD-PS) (data obtained from Fig. 3).

[c] The fraction of outer leaflet NBD-PS = the exchangeable NBD-PS pool (data obtained from Fig. 4).

[d] The fraction of outer leaflet NBD-PS = Tnp-NBD-PS/(Tnp-NBD-PS + NBD-PS).

vesicles, respectively. Derivatization of these LUV with TNBS (Table II) using 'non-penetrating' conditions yielded essentially the same results with the asymmetric outside-only and symmetrically labeled vesicles, but yielded inconsistent results with the asymmetric inside-only labeled population (see Discussion).

Mechanism of PS-decarboxylase decarboxylation

The foregoing results indicate that PS-decarboxylase can decarboxylate only NBD-PS localized in the outer leaflet of LUV. These observations raise the question as to whether the enzyme decarboxylates NBD-PS at the membrane surface or whether the preparation acts as a 'lipid acceptor' and decarboxylates the NBD-PS 'ex situ'. To determine which of these mechanisms might be operative, symmetric NBD-PS vesicles (60 nmoles) were incubated with enough PS-decarboxylase * (1 mg protein) to obtain a vesicle/PS-decarboxylase lipid ratio of 1:1 (see Materials and Methods). The PS-decarboxylase was then separated from the LUV by sucrose gradient centrifugation (samples made 50% sucrose and run on a 40% to 5% linear gradient) and the distribution of the fluorescent lipids was determined (Fig. 5a). Since approx. 25% of the fluorescence was found in the PS-decarboxylase fraction (at the bottom of the gradients), these results might indicate that PS-decarboxylase, can, under appropriate conditions, serve as a lipid acceptor and might suggest that PS-decarboxylase decarboxylates NBD-PS 'ex situ' (see Discussion). On the

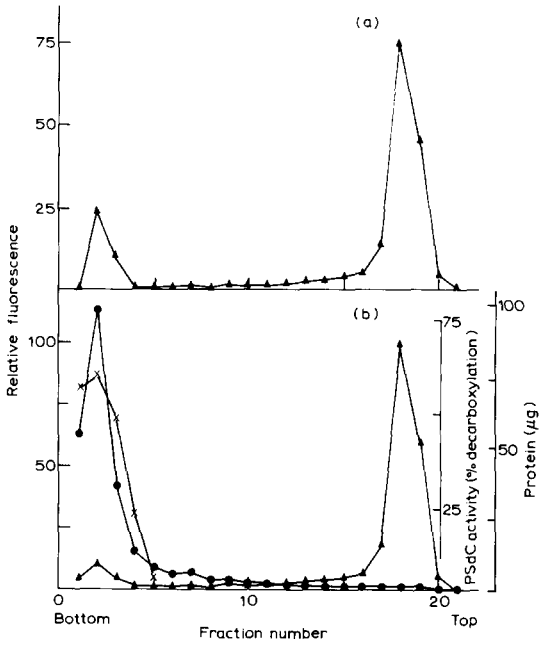

Fig. 5. Separation of LUV and PS-decarboxylase (PSdC) by sucrose gradient centrifugation. 60 nmoles LUV (DOPC/NBD-PS/^{125}I-PE, 99:1:trace) were incubated with 1.0 mg PS-decarboxylase (which contained 60 nmoles phospholipid) for 30 min at 37°C. The mixture was then made to 50% sucrose by adding solid sucrose. One ml of the mixture was overlayed with a linear 40% to 5% sucrose gradient (9 ml) and with 1 ml phosphate-buffered saline. The gradient was centrifuged in a SW 41 rotor at 38000 rpm for 2 h. Fractions (0.5 ml) were collected from the bottom of the tube and analyzed for protein (●), lipid (▲) and enzyme activity (×). Protein was determined according to Lowry and lipid by relative NBD fluorescence. PS-decarboxylase activity was determined from fractions obtained from a control gradient containing only PS-decarboxylase by incubating aliquots with NBD-PS dispersions for 1 min at 37°C.

* Since the fraction of lipid transfer is dependent on the relative concentration of the donor (NBD-PS containing LUV) and acceptor (PS-decarboxylase) populations, the ability to detect the fluorescent lipid in the PS-decarboxylase would be dependent on the presence of excess enzyme. THus, if one assumes that the affinity of the fluorescent lipid to the donor vesicles and to PS-decarboxylase are similar, at equal concentrations one would expect approx. 25% of fluorescent lipid (half of the outer leaflet) to reside with the PS-decarboxylase at equilibrium.

other hand, by employing small amounts of PS-decarboxylase (50 μg or less) efficient decarboxylation of PS does occur (see Fig. 2 and Table II) without significant redistribution/localization of the fluorescent product with the enzyme (Fig. 5b).

Discussion

The results of this study show that PS-decarboxylase efficiently decarboxylates NBD-PS in artificial bilayer membranes. Although this enzyme has previously been used for studying protein insertion into cytoplasmic membranes [21] it has never been used for generating asymmetric vesicles nor as an analytical technique for determining the degree of membrane asymmetry. Since it has been shown that PS-decarboxylase activity can be dependent on the source of the enzyme, on the acyl-chain of its substrate, and in some instances may even be inhibited by non-ionic detergents [33], our initial attempt to determine its applicability for the generation of asymmetric membranes and for the determination of the extent of PS asymmetry was directed towards assessing its activity on NBD-labeled analogs. The results of these experiments (see Fig. 1) showed that indeed PS-decarboxylase efficiently decarboxylated NBD-PS irrespective of the presence of detergent, and that it did not contain other lipases since no degradation of the other NBD analogs could be detected. In addition, the enzyme seemed to be stable in that its ability to decarboxylate could be reconstituted after lyophilization and did not appear to diminish after three months of storage at −20°C (results not shown).

Experiments employing unilamellar liposomes revealed that the enzyme could not decarboxylate more than approx. 50% of the NBD-PS present in a bilayer membrane. This suggested that the enzyme did not have access to lipid localized in the inner bilayer leaflet, nor did it induce bilayer translocation of NBD-PS from the inner to outer bilayer leaflet. This was verified through the use of asymmetric vesicles prepared by lipid transfer techniques, which showed that the enzyme decarboxylated all of the accessible NBD-PS in the outer leaflet of 'outside' LUV, but did not decarboxylate NBD-PS located in the inner-leaflet of 'inside' labeled populations. These results were further verified by two independent assays for determining the asymmetric distribution of lipids: (i) TNBS, which has been shown, under appropriate conditions, to preferentially derivatize aminophospholipids present in the outer leaflet of vesicles [32] and cells [3], and by (ii) resonance energy transfer, which has been shown to accurately assess the degree of NBD-labeled lipid asymmetry [29].

Although the results from these three assays correlated well, trinitrophenylation of vesicles containing only inner-leaflet NBD-PS resulted in an unexpected derivatization of approx. 50% of the NBD-PS (Table II). This result was possibly due to 'leaky' membranes induced by the previous removal of outer-leaflet NBD-PS, suggesting that TNBS penetrated the bilayer because of its low molecular weight, whereas PS-decarboxylase did not. The resonance energy transfer technique on the other hand, is an assay that is independent of membrane permeability characteristics.

Our results show that PS-decarboxylase can decarboxylate only NBD-PS localized in the outer leaflet of LUV. This observation raised the possibility that, as opposed to operating at the membrane surface, the enzyme may act as a lipid acceptor. Indeed, when large amounts of PS-decarboxylase were used, a substantial fraction of the fluorescence did colocalize with the enzyme (Fig. 5a). This result indicates that, under appropriate conditions, the enzyme can induce transfer of NBD-PS from the donor vesicles, suggesting that first enzyme-associated NBD-PS might be decarboxylated, followed by transfer of NBD-PE back into the 'donor' vesicles. We stress, however, that alternative mechanisms of decarboxylation are also possible. Indeed,when small amounts of PS-decarboxylase were used, $< 5\%$ of the total fluorescence colocalized with the separated PS-decarboxylase. Whatever the direct mechanism, it is clear that by employing appropriate amounts of enzyme, vesicles with an asymmetric distribution of NBD-PS and NBD-PE can be obtained.

Although, asymmetric NBD-lipid vesicles can be made by lipid transfer techniques [29] the preparation of such membranes is tedious, requires large amounts of acceptor vesicles that must be separated from the donor population by ultracentrifugation or column chromatography tech-

niques and is limited to producing vesicles with a single fluorescent lipid species. On the other hand, the use of PS-decarboxylase provides a convenient one-step method for generating vesicles containing outer-leaflet PE and inner-leaflet PS. In addition, PS-decarboxylase can also be employed in the determination of the transbilayer distribution of NBD-PS. Although the assay requires the use of appropriately labelled lipids, it is unlike direct chemical derivatization or other enzymatic techniques, in that it does not appear to induce membrane perturbations (such as those which might occur through the formation of lyso-lipids with phospholipase A_2 [34,35] – and it does not suffer from permeability problems encountered when using 'non-permeable' probes such as TNBS.

Acknowledgements

This work was supported in part by developmental Fund Grant 175416 from the University of Texas, M.D. Anderson Hospital and Tumor Institute at Houston and by National Institutes of Health Grant CA-40149.

References

1 Rothman, J.E. and Lenard, J. (1977) Science 195, 743–753
2 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–71
3 Gordesky, S.E., Marinetti, G.V. and Love, R. (1975) J. Membrane Biol. 20, 111–132
4 Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 323, 178–193
5 Deuticke, B., Poser, B., Lutkemeier, P. and Haest, C.W.M. (1983) Biochim. Biophys. Acta 731, 196–210
6 Bergman, W.L., Dressler, V., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 769, 390–398
7 Daleke, D.L. and Huestis, W.H. (1985) Biochemistry 24, 5406–5416
8 Bevers, E.M., Comfurius, P. and Zwaal, R.F.A. (1983) Biochim. Biophys. Acta 736, 57–66
9 Bevers, E.M., Comfurius, P., Van Rijn, J.L.M.L., Hemker, H.C. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122, 429–436
10 Chiu, D., Lubin, B. and Shohet, S.B. (1979) Br. J. Haematol. 41, 233–234
11 Lubin, B. Chiu, D., Bastacky, B., Roelofsen, B. and Van Deenen, L.L.M. (1981) J. Clin. Invest. 67, 1643–1649
12 Schwartz, R.S., Tanaka, Y., Fidler, I.J., Chiu, D., Lubin, B. and Schroit, A.J. (1985) J. Clin. Invest. 75, 1965–1973
13 Franck, P.F.H., Bevers, E.M., Lubin, B.H., Comfurius, P., Chiu, D.T.-Y., Op den Kamp, J.A.F., Zwaal, R.F.A., Van Deenen, L.L.M. and Roelofsen, B. (1985) J. Clin. Invest. 75, 183–190
14 Bretscher, M.S. (1973) in The Cell Surface in Development (Moscona, A.A., ed.), pp. 17–27, John Willey, New York
15 Zachowski, A., Favre, E., Cribier, S., Herve, P. and Devaux, P.F. (1986) Biochemistry 25, 2585–2590
16 Bishop, W.R. and Bell, R.M. (1985) Cell 42, 51–60
17 Kanfer, J. and Kennedy, E.P. (1963) J. Biol. Chem. 238, 2919–2922
18 Kanfer, J. and Kennedy, E.P. (1964) J. Biol. Chem. 239, 1720–1726
19 Dowhan, W., Wickner, W.T. and Kennedy, E.P. (1974) J. Biol. Chem. 249, 3079–3084
20 Clarke, L. and Carbon, J. (1976) Cell 9, 91–99
21 Tayhach, R.J., Hawrot, E., Stare, M. and Kennedy, E.P. (1979) J. Biol. Chem. 254, 627–633
22 Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 769–775
23 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400
24 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
25 Comfurius, P. and Zwaal, R.F.A. (1977) Biochim. Biophys. Acta 488, 36–42
26 Nayar, R. and Schroit, A.J. (1985) Biochemistry 24, 5967–5971
27 Schroit, A.J. (1982) Biochemistry 21, 5323–5328
28 Kremer, J.M.H., Van der Esker, M.W.J., Pathmamanoharan, C. and Wiersema, P.H. (1977) Biochemistry 16, 3932–3935
29 Pagano, R.E., Martin, O.C., Schroit, A.J. and Struck, D.K. (1981) Biochemistry 20, 4920–4927
30 Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099
31 Tanaka, Y. and Schroit, A.J. (1983) J. Biol. Chem. 258, 11335–11343
32 Litman, B.J. (1974) Biochemistry 13, 2844–2848
33 Verma, J.N. and Goldfine, H. (1985) J. Lipid Res. 26, 610–616
34 Bishop, D.G., Bevers, E.M., Van Meer, G., Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1979) Biochim. Biophys. Acta 551, 122–128
35 Weltzien, H.V. (1979) Biochim. Biophys. Acta 559, 259–287