Transbilayer Movement of Fluorescent Phospholipids in *Bacillus megaterium* Membrane Vesicles†

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ABSTRACT: We investigated the transbilayer movement or flip-flop of phospholipids in vesicles derived from the cytoplasmic membrane of *Bacillus megaterium*. Since common assay techniques were found to be inapplicable to the *Bacillus* system, we exploited and elaborated a newly described method in which fluorescent phospholipids (1-myristoyl-2-C_6-NBD phospholipids) are used as tracers to monitor flip-flop. These lipids were introduced into *Bacillus* vesicles from synthetic donor vesicles containing a fluorescence quencher. Transport was measured by monitoring the increase in fluorescence as the tracers departed the quenched environment of the donor vesicle and entered first the outer membrane leaflet and subsequently the inner leaflet of *Bacillus* vesicles. Independent experiments involving cobalt quenching of NBD fluorescence provided results consistent with the existence of pools of fluorescent phospholipid in the outer and inner leaflets of *Bacillus* vesicles at the completion of transport. Using the assay we show that phospholipid flip-flop in *Bacillus* vesicles occurs rapidly (half-time ∼30 s at 37 °C) with no preference for a particular phospholipid headgroup and that it is sensitive to proteolysis. We also establish that flip-flop does not occur in synthetic phospholipid vesicles or vesicles made from *Bacillus* phospholipids. We conclude that *Bacillus* vesicles possess the ability to promote rapid transbilayer movement of phospholipids, and that the transport is probably protein (flippase)-mediated.

The transbilayer movement or flip-flop of glycerophospholipids and other lipids is fundamental to the process of cellular membrane biogenesis and cell growth (Devaux, 1992; Menon, 1995; Zachowski, 1993). In bacteria, the enzymes of glycerophospholipid synthesis are located in the cytoplasmic (inner) membrane (Bell et al., 1971; Patterson & Lennarz, 1971; White et al., 1971) with their active sites oriented toward the cytosol. Newly synthesized phospholipids are thus initially located in the cytoplasmic leaflet of the bacterial inner membrane (Rothman & Kennedy, 1977a) and must be transported across the bilayer to populate the exoplasmic leaflet for membrane growth. Since flip-flop does not occur at an appreciable rate in protein-free membrane vesicles [*e.g.*, Kornberg and McConnell (1971)], it has been suggested that it is catalyzed to a biologically relevant rate by membrane proteins (termed flippases).

Early evidence of rapid transbilayer movement of phospholipids in biogenic membranes came from the work of Rothman and Kennedy (1977a) who used metabolic labeling and membrane topological analysis to show that phosphatidylethanolamine (PE)[1] traverses the cytoplasmic membrane bilayer of *Bacillus megaterium* and *Bacillus subtilis* rapidly (half-time for equilibration ∼1.5−3 min) and bidirectionally. Subsequent work using the same assay system showed that the transport appeared not to require any energy input and was independent of the synthesis of lipid and protein (Langley & Kennedy, 1979). Data published recently by Huijbregts et al. (1996) suggest that inner membrane vesicles from *Escherichia coli* behave similarly and are able to support rapid, bidirectional, energy-independent transbilayer movement of a variety of phospholipid analogues. Related observations have been made on eukaryotic endoplasmic reticulum (ER), a biogenic membrane system comparable to the cytoplasmic membrane of bacteria (Backer & Dawidowicz, 1987; Bishop & Bell, 1985; Buton et al., 1996; Hermann et al., 1990; Hutson & Higgins, 1985; van den Besselaar et al., 1978; Zilversmit & Hughes, 1977). Despite the documentation of rapid phospholipid flip-flop in these biogenic membrane systems, and a variety of persuasive (albeit indirect) evidence for protein involvement in this process, no biogenic membrane flippase has been isolated and the mechanism of catalyzed flip-flop remains to be described. The lack of progress in this area may, in part, be due to the fact that the flippase activity in biogenic

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[1] Abbreviations: BMV, *Bacillus megaterium* vesicle; BMV-PL, *B. megaterium* phospholipid vesicle; DV, donor vesicle; ER, endoplasmic reticulum; M-NBD-PC, 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycerol-3-phosphatidylcholine; M-NBD-PE, 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycerol-3-phosphatidylethanolamine; M-NBD-PG, 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycerol-3-phosphatidylglycerol; M-NBD-PL, 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycerol-3-phospholipid; NEM, *N*-ethylmaleimide; N-Rh-PE, L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (egg); PMSF, phenylmethanesulfonyl fluoride; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SV, synthetic acceptor vesicle.

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membranes is relatively unspecific with regard to phospholipid headgroup and cannot be conveniently stopped (say, for example, by depleting ATP or chilling samples on ice) for sample workup and analysis. This latter feature demands that assays for flippase activity in biogenic membranes must have very good time resolution, on the order of seconds rather than tens of seconds.

Bacillus spp. provide the opportunity to study a simple, accessible, flippase-active membrane system, similar in many ways to the biogenic endomembrane system (mainly ER) of eukaryotic cells. We therefore decided to investigate phospholipid flip-flop in *B. megaterium* with the eventual long-term aim of identifying membrane proteins with flippase activity. Our immediate objective was to confirm and expand on the observations of Rothman and Kennedy (1977a) by using a biochemically tractable flippase assay with adequate time resolution. Preliminary data indicated that standard assays used to examine flippase activity in animal cells, particularly those based on ultra-short-chain lipid substrates (Bishop & Bell, 1985; Rush & Waechter, 1995), "back-extraction" of lipid tracers with serum albumin (Buton et al., 1996), and dithionite reduction of NBD fluorophores in NBD-labeled phospholipids (McIntyre & Sleight, 1991; Ruetz & Gros, 1994; Williamson et al., 1995, Huijbregts et al., 1996), did not work in the *Bacillus* system. Instead we used a recently formulated fluorescence-based flippase assay (Zhang & Nichols, 1994) that exploits the characteristics of intermembrane transfer of short-chain phospholipids described previously by Nichols and Pagano (1981, 1982). We used short-chain fluorescent phospholipids as transport substrates, and monitored flipping continuously by fluorescence read-out in a fluorescence spectrophotometer. The principle transport parameters (rate constant and extent of transport) were then obtained by simple computer fitting of the kinetic data.

Using this assay we found that flip-flop of phospholipid analogs in right-side-out *B. megaterium* vesicles occurs extremely rapidly (half-time for equilibration ~30 s at 37 °C), and that it shows no phospholipid headgroup specificity. In addition we found that the transport rate was significantly reduced in protease-treated vesicles. The results indicate that *Bacillus* vesicles are endowed with the ability to promote rapid transbilayer movement of phospholipids and that the process is probably protein-mediated.

EXPERIMENTAL PROCEDURES

Materials and Routine Procedures. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), L-α-phosphatidylethanolamine-*N*-(lissamine rhodamine B sulfonyl) (egg) (N-Rh-PE), 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycerol-3-phosphatidylcholine (M-NBD-PC), 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycerol-3-phosphatidylethanolamine (M-NBD-PE), and 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycerol-3-phosphatidylglycerol (M-NBD-PG) were purchased from Avanti Polar Lipids (Alabaster, AL). Antibiotic Medium No. 3 was from Difco Laboratories (Detroit, MI), proteinase K and nuclease S7 were from Boehringer Mannheim (Indianapolis, IN), and lysozyme was from Sigma Chemical Co. (St. Louis, MO). Silica 60 thin-layer plates were from EM Science (Gibbstown, NJ). All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO).

Phospholipids were quantitated by measurement of lipid phosphorous according to Rouser et al. (1970) with inorganic phosphorous as standard. Fluorescent phospholipids were quantitated by measurement of the fluorescence in ethanol solution and comparison with similarly prepared standards. Protein concentrations were determined by the BCA method using the Micro BCA Protein Assay Reagent from Pierce Chemical Co. (Rockford, IL).

Preparation of Synthetic Donor and Acceptor Vesicles. Donor liposomes (DV) containing 3 mol % M-NBD-phospholipid, 6 mol % N-Rh-PE, and 91 mol % POPC were prepared by ethanol injection as described by Kremer et al. (1977). Briefly, the lipid mixture was dissolved in ethanol at a total lipid concentration of 10 mM, and the ethanol solution was injected slowly into 10 vol of 10 mM Tris, 0.1 M NaCl, pH 7.0, at room temperature while gently vortexing. The resulting donor liposomes were dialyzed overnight at 4 °C against 10 mM Tris, 0.1 M NaCl, pH 7.0, and used the same day. Acceptor vesicles were prepared similarly, except that no fluorescent phospholipids were included in the lipid mix.

Preparation of B. megaterium Membrane Vesicles (BMV). *B. megaterium* strain QM B1551 was grown in Penassay broth (Antibiotic Medium No. 3, Difco Laboratories) at 37 °C and harvested in log-phase growth by centrifugation. Membrane vesicles were prepared by lysozyme treatment in hypotonic buffer by a variation of the method described by Konings et al. (1973). Briefly, cells were harvested by centrifugation for 5 min at 10 000 rpm in a JA-20 rotor (Beckman) and washed once with ice-cold 50 mM potassium phosphate buffer, pH 8.0. The cells were then resuspended in the same buffer and diluted, while stirring, into 50 mM potassium phosphate, 10 mM $MgSO_4$ (pH 8.0) containing lysozyme (0.05 mg/mL) and nuclease S7 (20 units/mL). The suspension was stirred at 37 °C for an additional 30 min, and then 500 mM K-EDTA was added to a final concentration of 10 mM. After a 2 min incubation, 1 M $MgSO_4$ was added to a final concentration of 40 mM. The vesicles were collected via centrifugation for 45 min at 10 000 rpm (JA-20 rotor) and resuspended in 100 mM potassium phosphate, pH 6.5. The resuspended vesicles were subjected to low-speed centrifugation (2000*g* in a Sorvall RT6000D centrifuge) for 15 min, and the vesicles in the supernatant were collected by centrifugation for 15 min at 35 000 rpm (TLA 100.3 rotor, Beckman). Some preparations were subjected to additional low-speed centrifugations through 0.25 M sucrose in order to remove any remaining cell wall debris. These vesicles were resuspended in 50 mM potassium phosphate, pH 6.5, frozen immediately, and stored at −80 °C. Membrane vesicles made by this method have been shown to be sealed and entirely right-side-out oriented (Konings et al., 1973).

Preparation of Acceptor Vesicles from B. megaterium Phospholipids. *B. megaterium* was grown as described above, and lipids were extracted by the procedure of Bligh and Dyer (1959). The lipid extract was dissolved in chloroform and loaded onto an 8 mL silica gel column [silica gel, 70−230 mesh, 60 Å (Aldrich Chemical Co., Milwaukee, WI)]. The column was first washed with 4 column volumes of chloroform to remove neutral lipids; phospholipids were then eluted with 6 column volumes of chloroform/methanol, 1:1 (v/v). The phospholipid fraction was quantitated by determining total lipid phosphorous and analyzed by thin-

layer chromatography, using chloroform−methanol−water (65:25:4, by volume) as the mobile phase. TLC analysis showed that the phospholipid profile of the column-derived material was similar to that of the starting *B. megaterium* lipid extract. Acceptor vesicles were prepared by ethanol injection as described above, and termed *B. megaterium* phospholipid vesicles (BMV-PLs). TLC analysis showed that the phospholipid profile of BMV-PLs was similar to that of BMVs.

Flippase Assay Using Fluorescent Phospholipids. Donor liposomes containing the 1-myristoyl-2-C_6NBD-phospholipid of interest and a fluorescence quencher (N-Rh-PE) were prepared as described above. The donor liposomes (2.5 μL, ~2 nmol of phospholipid) were added to a cuvette containing 2 mL of 10 mM Tris, 0.1 M NaCl (pH 7.0), pre-incubated at 37 °C in the temperature-controlled stage of an SLM 8000 fluorescence spectrophotometer. The cuvette was equipped with a magnetic stir bar and the sample was stirred throughout the measuring period. Fluorescence [excitation 475 nm (4 nm bandpass), emission 530 nm (16 nm bandpass)] was monitored at 0.5 s intervals. After about 30 s (during which a constant low-fluorescence signal was recorded), acceptor vesicles [synthetic acceptors (POPC vesicles; SVs) or *Bacillus megaterium* vesicles (BMVs); usually ~5−20 μL corresponding to ~3−20 nmol of phospholipid, see figure legends and tables for precise amount used in a particular experiment] were added by injection through a rubber septum in the lid of the cuvette holder. Introduction of the acceptor vesicles prompted a sharp increase in fluorescence followed by a second slower increase (in the case of *B. megaterium* vesicles) or a plateau (POPC vesicles or BMV-PLs). Data were collected for a total time of at least 100 s (including the initial 30 s period). The fluorescence increase was fit to equations, with one or two exponential terms, of the general form

$$F(t) = A_1[1 - \exp(-k_1 t)] + A_2[1 - \exp(-k_2 t)] + F_0$$

where $F(t)$ is the fluorescence as a function of time and F_0 is the fluorescence of NBD-lipid at time = 0 s (*i.e.*, fluorescence of the NBD-lipid in donor vesicles), k_1 and k_2 are the rate constants for the fast and slow phases, respectively, and A_1 and A_2 are the amplitudes of the fast and slow phases, respectively. Curve fitting was performed with a non-linear least squares regression analysis using the Prism software package (GraphPad Software Inc.).

Protease Treatment. *B. megaterium* membrane vesicles were incubated with proteinase K (2.2 mg/mL) at room temperature for 30 min. Proteolysis was stopped by adding 4 mM PMSF (added from a 150 mM stock solution prepared in isopropyl alcohol; the final concentration of isopropyl alcohol in the sample was 2.6%). Control samples included vesicles treated with PMSF alone or vesicles incubated in buffer only (mock-treated).

NEM Treatment. *B. megaterium* membrane vesicles were incubated with 2 or 20 mM *N*-ethylmaleimide (NEM) in 75 mM potassium phosphate buffer (pH 7.4) for 30 min at 37 °C. Samples were stored on ice after the incubation and assayed within 30 min. Control samples were incubated similarly in buffer without NEM.

Cobalt Quenching Experiments. Samples were prepared as for a flippase assay described above except that the measurement was performed at 7 °C rather than 37 °C. BMVs, SVs, or *B. megaterium* phospholipid vesicles (BMV-PLs) were added to a cuvette containing DVs, and the sample was incubated until the fluorescence signal was seen to reach a plateau (~10 min in the case of BMVs). $CoCl_2$ was then injected into the cuvette from a 1 or 2 M stock solution in water, to give a final concentration of 0.0125−30 mM, and the decrease in fluorescence was monitored. The new level of fluorescence intensity (termed F_{obs} below) was noted for each concentration of $CoCl_2$, and the value was corrected for inner filter effects using the equation

$$F_{corr} \approx F_{obs} \times 10^{[(OD_{EX} + OD_{EM})/2]}$$

(Lakowicz, 1983) where F_{obs} is the measured fluorescence intensity in the presence of quencher, OD_{EX} is the optical density of $CoCl_2$ at the excitation wavelength (475 nm), and OD_{EM} is the optical density of $CoCl_2$ at the emission wavelength (530 nm). The corrected values were analyzed according to the Stern−Volmer law:

$$F_o/F = 1 + K_{SV}[Q]$$

where F_o is the fluorescence intensity in the absence of $CoCl_2$, F is the corrected fluorescence intensity in the presence of $CoCl_2$, K_{SV} is the Stern−Volmer quenching constant, and [Q] is the concentration of quencher.

Metabolism. BMVs were incubated with DVs as in the flippase assay for 5 min at 37 °C. BMVs were separated from the DVs by centrifugation [15 min, 4 °C, 35 000 rpm. TLA100.3 rotor (Beckman)] through a 1 mL cushion of 0.5 M sucrose. The BMV pellet was resuspended in 250 μL of water and extracted by the procedure of Bligh and Dyer (1959). The lipid extract was analyzed by thin layer chromatography, using chloroform−methanol−water (65:25:4, by volume) as the mobile phase. The only NBD-fluorescent species seen corresponded to the NBD-phospholipid used in the DV; there was no evidence of monoacyl or neutral fluorescent species.

Mathematical Model for the Transport Assay. A three-pool model describing the principal features of the transport assay is shown schematically in Figure 7A. M-NBD-phospholipids in the outer leaflet of DVs (pool a, containing q_a molecules of fluorescent lipid) equilibrate with lipid pools in the outer leaflet of BMVs (pool b, containing q_b molecules) and in the inner leaflet pool of BMVs (pool c, containing q_c molecules). The three-pool model can be applied to our specific model of lipid transfer through the aqueous phase (see Figure 1, step 1) by assuming that the concentration of fluorescent lipid in the aqueous phase reaches equilibrium rapidly. Differential equations describing the time evolution of each of the three pools are as follows:

$$dq_a/dt = -k_{ab}q_a + k_{ba}q_b \quad (1)$$

$$dq_b/dt = k_{ab}q_a - (k_{ba} + k_{bc})q_b + k_{cb}q_c \quad (2)$$

$$dq_c/dt = k_{bc}q_b - k_{cb}q_c \quad (3)$$

where k_{ab}, k_{ba}, k_{bc}, and k_{cb} are the rate constants for transport from pool a to pool b, pool b to pool a, pool b to pool c, and pool c to pool b, respectively (see Figure 7A).

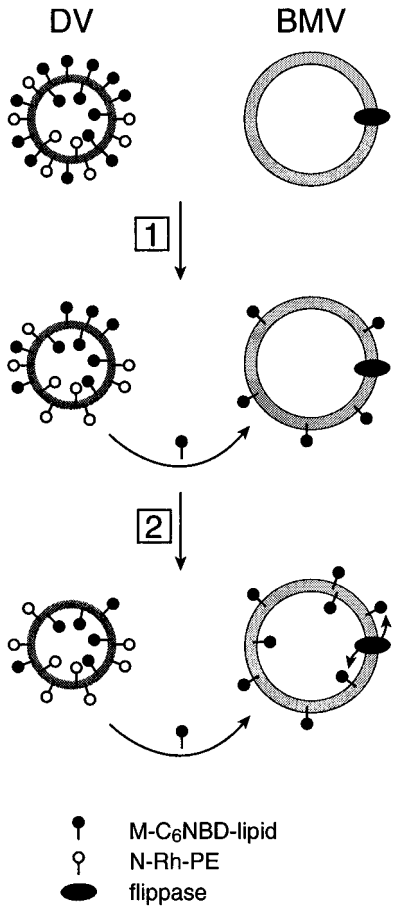

FIGURE 1: Outline of the flippase assay. Step 1, interbilayer transfer; step 2, transbilayer transfer of M-NBD-phospholipid. DV, donor vesicle; BMV, *B. megaterium* vesicle. Phospholipids are represented by open or filled circles (headgroup) appended to a short line (lipid moiety).

The solutions to these equations are of the general form

$$q_a/q_{a0} = H_1 \exp(-g_1 t) + H_2 \exp(-g_2 t) + H_3 \exp(-g_3 t) \quad (4)$$

$$q_b/q_{a0} = K_1 \exp(-g_1 t) + K_2 \exp(-g_2 t) + K_3 \exp(-g_3 t) \quad (5)$$

$$q_c/q_{a0} = L_1 \exp(-g_1 t) + L_2 \exp(-g_2 t) + L_3 \exp(-g_3 t) \quad (6)$$

where q_{a0} is the amount of transportable fluorescent lipid in the assay and H_1, H_2, H_3, K_1, K_2, K_3, L_1, L_2, L_3, g_1, g_2, and g_3 are constants (see below).

The fluorescence traces obtained in the transport assay are proportional to the amount of M-NBD-phospholipid transferred to the acceptor vesicles. In order to use eq 4 to calculate the rate constants for the transfer reaction, fluorescence traces were transformed to describe the fraction of the total M-NBD-phospholipid lost from the DVs, *i.e.*, lost from pool a. This was accomplished by determining the maximum fluorescence obtained when all of the probe was transferred to the BMVs (F_{max}); the fraction of maximum fluorescence transferred to the acceptors is thus given by $F(t)/(F_{max} - F_o)$. Since no probe is lost during the transfer reaction, and assuming that the fluorescence yield of M-NBD-phospholipid is the same in both leaflets of the BMV bilayer, the fraction of transportable lipid in the donors (q_a/q_{a0}) is equal to $1 - F(t)/(F_{max} - F_0)$. The transformed data were used to obtain the parameters H_1, H_2, H_3, g_1, and g_2 from the best fit to eq 4. The rate constant g_3 is equal to zero since no probe is lost from the three pools. The rate constants k_{ab}, k_{ba}, k_{bc}, and k_{cb} were calculated from these parameters using the algorithm presented on page 48 in Shipley and Clark (1972). These parameters and rate constants were used to calculate K_1, K_2, K_3, L_1, L_2, and L_3 (Shipley & Clark, 1972) which allowed the projection of the fractional amount of M-NBD-phospholipid in pools b and c as a function of time (Figure 7B). The equilibrium distribution of fluorescent lipid in the two leaflets of BMVs is given by outer leaflet/inner leaflet $= q_b/q_c = k_{cb}/k_{bc}$.

RESULTS

Flippase Assay

The flippase assay used in this study is illustrated schematically in Figure 1. The assay detects two transport steps: (1) transfer of a fluorescent phospholipid (the flippase substrate) from the outer leaflet of "donor" vesicles (DVs) to the outer leaflet of "acceptor" vesicles derived from the cytoplasmic membrane of *B. megaterium* (BMVs, Figure 1, step 1) and (2) flippase-catalyzed transfer of the phospholipid across the BMV bilayer into the inner membrane leaflet (Figure 1, step 2). We were able to distinguish and separately characterize the intermembrane and transbilayer transport events by using fluorescent 1-myristoyl-2-C_6-(NBD)-phospholipids (M-NBD-PLs) as transport substrates. These phospholipids contain relatively short fatty acyl chains and are consequently considerably more water soluble than their naturally occurring counterparts. As a result of their enhanced water solubility, M-NBD-PLs can equilibrate between the outer leaflets of donor and acceptor vesicle membranes very rapidly with a half-time of only a few seconds (<3 s) at 37 °C (Figure 4A), much faster than any reported half-time for transbilayer translocation of phospholipids in bacterial membranes [half-times of 1.5−3 min in *B. subtilis* and *B. megaterium* (Rothman & Kennedy, 1977a), and 7 min in *E. coli* inner membrane vesicles (Huijbregts et al., 1996) at 37 °C]. We exploited this kinetic separation of the intermembrane and transbilayer transfer processes to study transbilayer movement (flipping) of M-NBD-PLs in BMVs.

DVs were prepared from phosphatidylcholine, trace amounts of an M-NBD-PL (the transport substrate), and trace amounts of N-Rh-PE (a fluorescent phospholipid able to quench NBD fluorescence in the same vesicle but unable to transfer between vesicles). NBD fluorescence in DVs was low owing to the presence of N-Rh-PE quenchers (Figure 2). Addition of acceptor vesicles (DVs or BMVs) resulted in transport and equilibration of M-NBD-PLs between the DVs and acceptor vesicles, and a concomitant increase in the fluorescence signal due to non-quenched M-NBD-PL molecules newly located in the acceptors. When BMVs were used as acceptors, a biphasic fluorescence increase was observed (Figure 2, trace marked BMV) that could be deconvoluted by a double exponential fit of the data (Figure 2, BMV fit). We suggest that the first phase corresponds to transfer of the M-NBD-phospholipid (in this case M-NBD-PE) from the outer leaflet of DVs to the outer leaflet of BMVs (rate constant $k_1 = 0.296\ s^{-1}$, half-time = 2.3 s), and the second

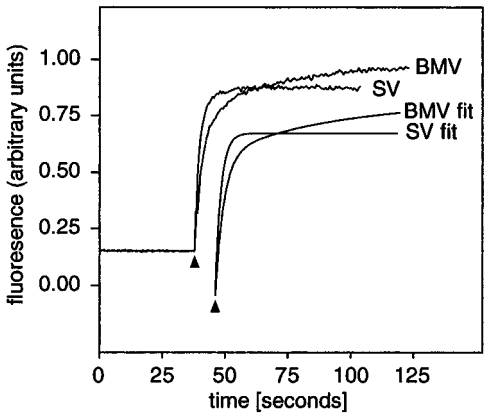

FIGURE 2: Transport of M-NBD-PE from DVs to acceptor vesicles. SV and BMV traces were generated from separate measurements at 37 °C as described in Experimental Procedures. The arrowhead indicates the point at which acceptor vesicles (SVs or BMVs) were introduced into the measuring cuvette. The SV trace could be described by the following single exponential equation: $F(t) = 0.717[1 - \exp(-0.449t)]$. The BMV trace, however, showed two phases that could only be well described by a double exponential equation: $F(t) = 0.617[1 - \exp(-0.296t)] + 0.238[1 - \exp(-0.022t)]$. For both fits, F_o was set arbitrarily to zero. The single and double exponential fits (SV fit and BMV fit, respectively) are shown displaced from the experimental traces for clarity.

phase corresponds to slower transbilayer movement of M-NBD-PE in the BMVs with a half-time of ~31 s (rate constant $k_2 = 0.022$ s^{-1}). This proposal implies that the second fluorescence increase is caused by additional transfer of phospholipids from DVs to BMVs in response to the translocation of M-NBD-phospholipids from the BMV outer leaflet to the BMV inner leaflet. Since the transbilayer translocation rate is slower than the intermembrane transfer rate, the second phase can be explicitly characterized, separate from the first phase, and its kinetics can be taken to be indicative of the transbilayer translocation rate. Additional data for M-NBD-PE transport are summarized in Table 1.

The two rate constants (k_1 and k_2) differ by an order of magnitude and therefore provide good estimates of the intermembrane and transbilayer rate processes. The improved time resolution of this assay (limited only by a sample mixing time of a few seconds since data are acquired every 0.5 s) most likely accounts for the shorter half-time measured here for transbilayer movement compared to previous reports based on slower assay techniques (Rothman & Kennedy, 1977a; Huijbregts et al., 1996). When flippase-inactive synthetic "acceptor" vesicles (SVs) or vesicles made from *Bacillus* phospholipids (BMV-PLs) were used as acceptors, only a monophasic fluorescence increase was observed (Figure 2, traces marked SV and SV fit; BMV-PL data not shown) corresponding solely to intermembrane transfer.

Characteristics of the Rate and Extent of Intermembrane and Transbilayer Transfer of M-NBD-Phospholipid

The kinetics of interbilayer transfer have been previously shown to depend on a number of parameters, including the characteristics of the NBD-phospholipid, temperature, phospholipid composition of the donor and acceptor membranes, and the donor/acceptor ratio (Nichols & Pagano, 1982). In order to test our suggestion that the fast-phase rate constant (k_1) describes interbilayer transport, we examined its behavior (i) at different temperatures and (ii) as a function of the donor/acceptor ratio using compositionally identical donor and acceptor pairs (DVs and SVs) and compositionally different donor and acceptor pairs (DVs and BMVs).

The magnitude of k_1 for M-NBD-PE transfer between DVs and SVs was found to depend on temperature as shown in the Arrhenius plot in Figure 3A. The interbilayer rate constant (k_1) for transfer to BMVs showed a similar temperature dependence (see Figure 3B), but the transbilayer rate constant (k_2) appeared to be largely independent of temperature between 20−41 °C.

The half-time of interbilayer transfer [$t_{1/2} = (\ln 2)/k_1$] was independent of the donor/acceptor ratio when similar donors and acceptors were used (DVs and SVs) (Figure 4A, open squares) but were clearly dependent on the donor/acceptor ratio when compositionally disparate donors and acceptors were used (DVs and BMVs) (Figure 4A, filled circles). The half-time of M-NBD-PE transfer between the compositionally dissimilar DVs and BMVs approached the half-time for transfer between similar vesicles (DVs and SVs) as the donor/acceptor ratio approached zero (Figure 4A). This result is predicted from the model of Nichols and Pagano (1982) which describes monomer diffusion of amphiphiles between membrane vesicles. The data and model predict that the dissociation rate constant for the DVs (0.55 s^{-1}) is roughly twice that for the BMVs (0.25 s^{-1}).

In contrast to the behavior of the half-time for interbilayer transfer, the half-time corresponding to the second kinetic phase observed for M-NBD-PE transfer from DVs to BMVs was independent of the DV/BMV ratio (Figure 4B), as would be expected for a process limited to the acceptor membrane. This is consistent with the proposal that the rate constant k_2 describes transbilayer movement in BMVs.

Table 1: Interbilayer and Transbilayer Transport of M-NBD-Phospholipids[a]

	SV acceptors	BMV acceptors			
phospholipid	k_1 (s^{-1})[b]	k_1 (s^{-1})[c]	k_2 (s^{-1})	A_1	A_2
M-NBD-PE	0.555 ± 0.075 (20)	0.271 ± 0.033 (7)	0.024 ± 0.002 (7)	0.511 ± 0.081 (7)	0.224 ± 0.014 (7)
M-NBD-PC	0.910 ± 0.257 (18)	0.386 ± 0.020 (5)	0.026 ± 0.003 (5)	0.872 ± 0.386 (5)	0.322 ± 0.146 (5)
M-NBD-PG	0.768 ± 0.152 (16)	0.459 ± 0.081 (6)	0.026 ± 0.006 (6)	0.382 ± 0.091 (6)	0.193 ± 0.037 (6)

[a] The data are presented as mean ± standard deviation (number of determinations); k_1 and k_2 are defined in the Experimental Procedures; measurements were performed at 37 °C. [b] k_1 values for transfer between compositionally identical donor and acceptor vesicles (such as DVs and SVs) are independent of the donor/acceptor ratio; the rate constants tabulated represent data averaged over many experiments conducted using a range of donor/acceptor ratios. The transport amplitudes are, however, dependent on the donor/acceptor ratio and, for a DV/SV phospholipid ratio of 0.22, transport amplitudes for the different fluorescent phospholipids were as follows: 0.564 ± 0.066 (12), 0.574 ± 0.363 (9), and 0.721 ± 0.258 (7) for M-NBD-PE, M-NBD-PC, and M-NBD-PG, respectively. Amplitudes are presented as arbitrary fluorescence units. [c] k_1 values for transfer of M-NBD-phospholipids from DVs to BMVs are dependent on the donor/acceptor ratio as shown in Figure 4A. The values given here correspond to a donor/acceptor (DV/BMV) phospholipid ratio of 0.22.

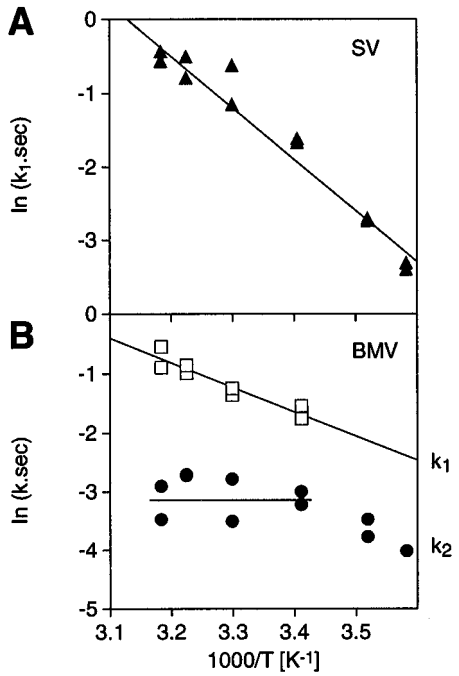

FIGURE 3: Temperature dependence of the rate constants. (A) Arrhenius plot of the interbilayer rate constant (k_1) for transfer of M-NBD-PE between DVs and SVs, with POPC donor vesicles and POPC acceptors at a donor/acceptor phospholipid ratio of 0.25. (B) Arrhenius plot of the interbilayer and transbilayer rate constants (k_1 and k_2) for transfer of M-NBD-PE between DVs and BMVs (donor/acceptor phospholipid ratio of 0.11). Measurements were performed over a temperature range of 6−41 °C.

The amplitudes of the two kinetic phases correlate with the equilibrium distribution of the fluorescent lipid between DV and BMV membranes. The amplitude for the fast kinetic phase (A_1) decreased as the acceptor population decreased relative to donor (for both SV and BMV; SV data not shown) up to a certain level at which point the NBD-phospholipid became limiting (shown for BMV in Figure 4C); also, predictably, the amplitude of the slow phase (A_2) increased as the donor/acceptor ratio increased (Figure 4C).

Specificity of Transbilayer Movement

We investigated the specificity of transbilayer phospholipid movement in BMVs. The results are summarized in Table 1. The three phospholipids studied (M-NBD-PE, M-NBD-PC, M-NBD-PG) showed similar rates of transbilayer movement (k_2 = 0.024−0.026 s^{-1}, corresponding to half-times of 26.5−28.7 s) compatible with data obtained with other biogenic membrane preparations (Buton et al., 1996; Hermann et al., 1990; Huijbregts et al., 1996). The precise value for k_2 varied somewhat between vesicle preparations, and although the value was typically ∼0.025 s^{-1} it was as high as ∼0.055 s^{-1} (corresponding to a half-time of ∼12 s) in some BMV preparations.

The rate of interbilayer transport (k_1) varied depending on the phospholipid headgroup. This can be seen in Table 1 for transfer to SV and BMV acceptors. For synthetic vesicle (SV) acceptors, the rate of interbilayer equilibration decreased predictably (Nichols & Pagano, 1982) with headgroup in the order M-NBD-PC > M-NBD-PG > M-NBD-PE. However, for BMV acceptors the order was M-NBD-PG > M-NBD-PC > M-NBD-PE; the change in order and the difference in magnitude is most likely related to the different phospholipid composition of the BMVs versus the SVs (Nichols & Pagano, 1982).

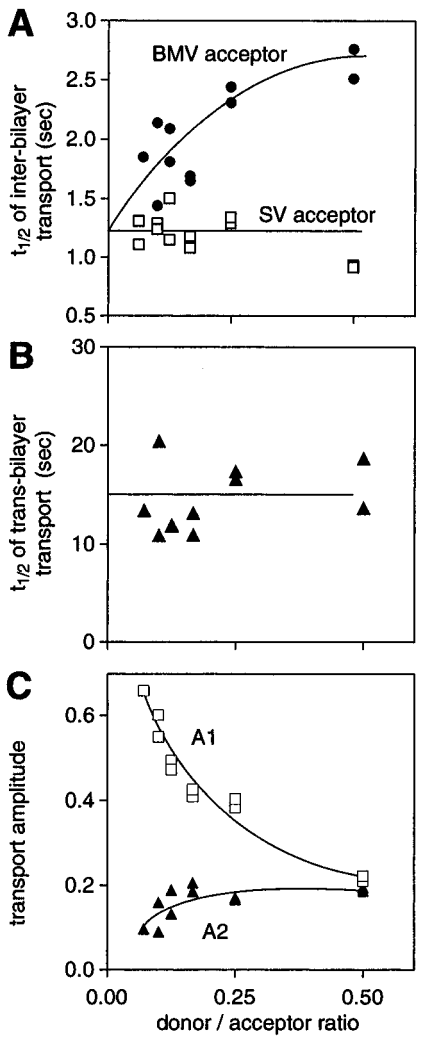

FIGURE 4: Characteristics of interbilayer and transbilayer transport of M-NBD-PE. (A) Plot of the half-time [$t_{1/2}$ = (ln 2)/k_1] of interbilayer transfer for M-NBD-PE versus the donor/acceptor vesicle ratio. M-NBD-PE transfer was from POPC donors to BMV acceptors or SV acceptors. (B) Plot of the half-time [$t_{1/2}$ = (ln 2)/k_2] of transbilayer transfer for M-NBD-PE in BMV acceptors versus the donor/acceptor ratio. (C) Plot of the amplitudes of interbilayer (A_1) and transbilayer (A_2) transfer of M-NBD-PE in BMV acceptors versus the donor/acceptor ratio. Donor/acceptor ratios are presented operationally as ratios of the volumes of vesicle suspensions used in the assay. DV and SV suspensions were prepared at 2 nmol of lipid phosphorus per 2.5 μL; the BMV preparation used contained 0.61 nmol of lipid phosphorus per μL. All measurements were performed using 2.5 μL of DVs per assay.

Effect of Protein Modification Reagents on Flippase Activity

The effect of protease treatment on interbilayer and transbilayer transport was examined. A representative experiment with M-NBD-PE is shown in Figure 5, and the cumulative data are summarized in Table 2. The interbilayer rate constant and amplitude (k_1 and A_1) were similar for protease-treated vesicles and controls. In contrast, protease treatment resulted in a ∼50% decrease in the transbilayer rate constant (k_2) without affecting the second phase ampli-

Table 2: Effect of Protease Treatment on Interbilayer and Transbilayer Transport of M-NBD-Phospholipids in *B. megaterium* Membrane Vesicles[a]

treatment	phospholipid	k_1 (s^{-1})	k_2 (s^{-1})	A_1	A_2
mock	M-NBD-PE	0.288 ± 0.025	0.021 ± 0.0001	0.302 ± 0.013	0.172 ± 0.017
PMSF		0.332 ± 0.078	0.023 ± 0.002	0.321 ± 0.008	0.155 ± 0.006
proteinase K		0.291 ± 0.012	0.012 ± 0.003	0.341 ± 0.007	0.183 ± 0.020

[a] All data are provided as mean ± standard deviation of three determinations, using donor vesicles and BMV acceptors as described in Experimental Procedures. The BMV acceptors were either mock treated, treated with PMSF, or treated with proteinase K followed by PMSF, before testing. Measurements were performed at a donor/acceptor (DV/BMV) phospholipid ratio of 0.27. Amplitudes are presented as arbitrary fluorescence units.

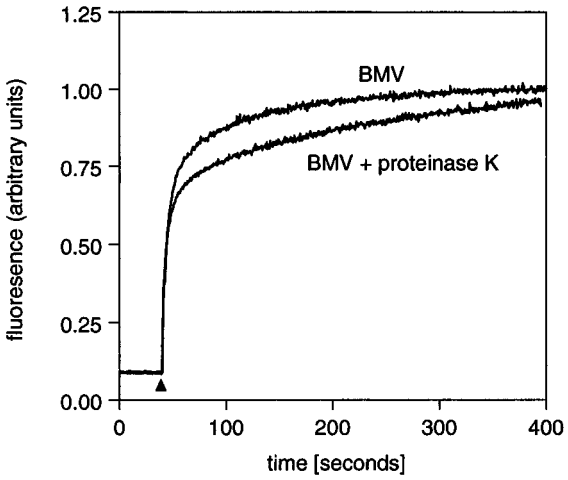

FIGURE 5: Effect of protease treatment on transbilayer movement. The traces were generated from separate measurements at 37 °C, using M-NBD-PE-containing DVs and control or protease-treated BMVs as described in the Experimental Procedures. BMVs were incubated with proteinase K for 30 min at 37 °C and proteolysis was stopped with PMSF. A control sample was incubated similarly in buffer without proteinase K. The arrowhead indicates the point at which BMVs were introduced into the measuring cuvette. PMSF alone had no effect on the measurement. These and additional data on the effect of proteinase K treatment are summarized in Table 2.

tude (A_2). This was true for both M-NBD-PE (Table 2) and M-NBD-PC (data not shown). Thus protease treatment lowered the rate of transbilayer transport without affecting the amount transported. These data cannot be attributed to loss of vesicle (BMV) integrity as a result of protease treatment since the predicted outcome of the experiment in compromised vesicles would be an increase in the second rate constant (approaching k_1), with no change in the total amount transported.

NEM treatment had no effect on transport of M-NBD-PE. Parameters for interbilayer and transport were similar in NEM-treated and control samples (data not shown).

Quenching of NBD Fluorescence by Divalent Cobalt Ions

The kinetic analysis of M-NBD-PL transport presented above implies the existence of two pools of M-NBD-PL molecules associated with the BMV acceptors at the completion of the transport assay. We have suggested that the two pools correspond to M-NBD-PLs located in the external and internal leaflets of the BMV membrane bilayer, and that the internal leaflet pool is populated as a consequence of transbilayer movement of M-NBD-PLs from the external leaflet (Figure 1). In the experiments described in this section, we use a fluorescence quenching approach to provide separate evidence of the existence of two pools of fluorescent M-NBD-PLs in BMVs.

NBD fluorescence can be quenched by divalent cobalt ions (Morris et al., 1985) via a collisional quenching mechanism dictated by the Stern−Volmer law (Lakowicz, 1983). When solutions of cobalt chloride were added to an equilibrated flippase assay mixture, *i.e.*, after the distribution of M-NBD-PL molecules between BMVs and DVs had reached an equilibrium and net transport was complete, the fluorescence signal rapidly dropped to a new equilibrium value that was dependent on the cobalt concentration used (up to ∼10 mM). No effect was observed if Ca^{2+} was used instead of Co^{2+}. Measurements were carried out at 7 °C to reduce the possibility of leakage of cobalt ions across the BMV membrane, since experiments conducted at 25 °C showed a slow downward drift of the cobalt-quenched signal, consistent with leakage of cobalt ions into the vesicle interior. Representative traces from quenching experiments performed with M-NBD-PE in BMVs at 7 °C are shown in Figure 6A. Data from two such experiments with BMVs were transformed according to the Stern−Volmer equation to generate the BMV trace shown in Figure 6B.

The Stern−Volmer plot for M-NBD-PE in BMVs (Figure 6B, trace marked BMV) is curved, and plateaus at $F_o/F = 2.5$. The curvature of the plot for BMV acceptors suggests the existence of two fluorescent pools of M-NBD-PE in the assay system (Lakowicz, 1983), and the plateau value of 2.5 indicates that 40% of the M-NBD-PE molecules are inaccessible to cobalt ions. These data are most simply interpreted by suggesting that upto 60% of the M-NBD-PE molecules are located in the BMV outer leaflet and consequently available for cobalt quenching, whereas at least 40% are located in the inner leaflet and inaccessible to cobalt.

The interpretation of these results is, however, subject to a number of complicating factors. For example, since collisional quenching of NBD fluorescence in BMVs probably occurs through vesicle surface-associated cobalt ions bound to negatively charged BMV phospholipids (Morris et al., 1985) (phosphatidylglycerol and cardiolipin; the Stern−Volmer quenching constant (K_{SV}) obtained from the BMV data shown in Figure 6B was ∼1000 M^{-1}, much greater than the quenching constant for M-NBD-PE in SVs prepared from phosphatidylcholine [$K_{SV} \sim 40$ M^{-1}, Figure 6B, trace marked SV)], the results discussed above could also be interpreted by proposing that the quenching plateau observed with BMVs is due to saturation of cobalt ion binding sites on the BMV surface. Results obtained with BMV-PLs argue against this interpretation. The Stern−Volmer plot for these flippase-inactive vesicles (Figure 6B, trace marked BMV-PL) shows an initial quenching efficiency similar to that for BMVs, but no quenching plateau. The slight curvature of the BMV-PL trace suggests the existence of at least two pools of NBD-phospholipid in BMV-PLs (possibly corresponding to two conformations of the NBD-

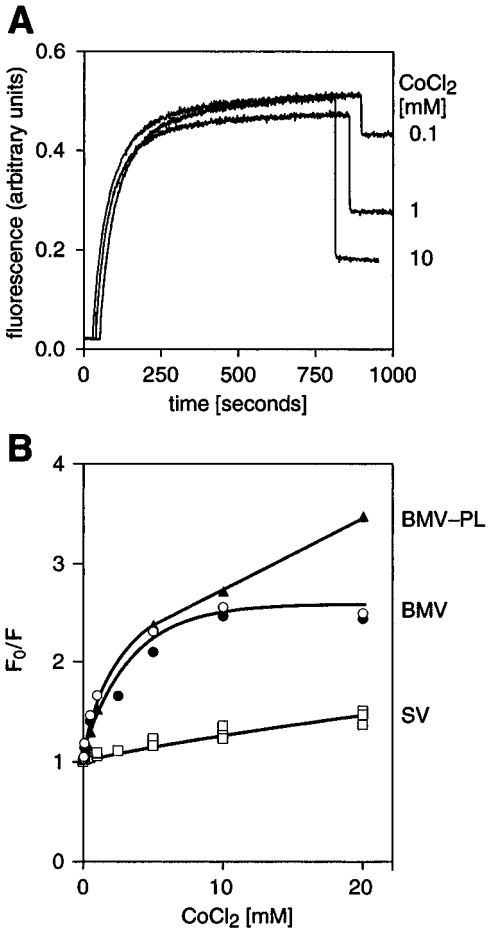

FIGURE 6: Quenching of M-NBD-PE fluorescence in BMVs by cobalt ions. Samples were prepared as for a flippase assay (see Experimental Procedures and Figure 2), except that the measurement was performed at 7 °C. Acceptor vesicles were added to a cuvette containing DVs and the sample was incubated until the fluorescence signal was seen to reach a plateau (~10 min). $CoCl_2$ was then injected into the cuvette from a 1 or 2 M stock solution in water, to give a final concentration of 0.0125−30 mM, and the decrease in fluorescence was monitored. (A) Representative fluorescence traces showing the approach to equilibrium in transport assay with BMVs and the subsequent reduction in fluorescence following addition of $CoCl_2$. The time of cobalt addition corresponds to the sharp drop in fluorescence in each case. (B) Stern−Volmer plot based on the corrected fluorescence intensity (see Experimental Procedures); the open and filled circles used in the BMV trace represent two different experiments with BMVs; the triangles represent measurements with *Bacillus* phospholipid vesicles, and the open squares represent measurements with phosphatidylcholine SVs.

containing acyl chain), both of which are accessible to cobalt ions albeit with slightly different efficiency.

DISCUSSION

The fluorescence assay we describe takes advantage of the observation that certain fluorescent phospholipids can transfer between membranes extremely rapidly with half-times on the order of 1 s, but that transbilayer movement occurs on a time scale of tens of seconds. Thus fluorescent phospholipids contained in the outer leaflet of "donor" vesicles can transfer rapidly into the outer leaflet of flippase-active or inactive "acceptor" vesicles in a single kinetic event, the equilibrium between donor and acceptor being reached in a matter of seconds (Figure 1, step 1). Transbilayer movement would then occur in flippase-active vesicles, displacing the first equilibrium and giving rise to a second event with characteristically slower kinetics.

In our experimental set-up, 1-myristoyl-2-C_6NBD-phospholipids originate in donor vesicles containing a quencher of NBD fluorescence and give rise to fluorescence increases as transport to non-quenching acceptor vesicles occurs. Intermembrane transfer occurs by monomer diffusion of the NBD-lipid from the outer leaflet of the donor liposome to the outer leaflet of the acceptor liposome (Nichols & Pagano, 1981, 1982; Figure 1); the monomer intermediate in the aqueous phase is not detected as the NBD fluorophore is essentially non-fluorescent in water (Chattopadhyay, 1990). When BMVs are used as acceptors, two kinetically discrete fluorescence increases are observed; when SVs or *Bacillus* phospholipid vesicles are used as acceptors, only the initial fluorescence increase is detected (Figure 2). Thus it is absolutely clear that the presence of BMVs in the assay results in two kinetic events, corresponding to two distinct pools of fluorescent phospholipid. Separate fluorescence quenching experiments (Figure 6) also indicate the presence of two pools of fluorescent lipid in the BMVs, one pool being inaccessible to cobalt ions and therefore most likely to be located in the inner leaflet of the BMV membrane bilayer.

The BMV-specific, second, slower kinetic phase of the fluorescence assay is therefore most simply and plausibly interpreted as the consequence of transbilayer movement. The corresponding rate constant (k_2), unlike k_1, is not affected by changing the ratio of donor to acceptor vesicles (Figure 4B), consistent with the proposal that it describes an event confined to the acceptor membrane. Furthermore, the temperature dependence of k_2 is completely distinct from that of k_1. These observations also argue against the possibility that the two kinetic phases observed with BMVs could be the result of a difference in the rate of interbilayer transfer caused by size or compositional heterogeneity in the population of BMVs. Furthermore, alternative explanations for the second kinetic phase such as slow nonspecific adsorption to a vesicle surface protein(s) are ruled out by the cobalt quenching experiments alluded to above (Figure 6) and the protease treatment experiments summarized in Table 2. Proteinase K treatment results in a decrease in the slow rate constant (k_2) without affecting the slow-phase amplitude (A_2); the amplitude would be expected to be lowered if the M-NBD-phospholipids were being slowly adsorbed to vesicle proteins following transfer into the outer leaflet of the BMVs.

As pointed out briefly in the Introduction to this paper, commonly used techniques for the measurement of flippase activity (Menon, 1995) did not work in the *Bacillus* system. For example, we were unable to "back-extract" fluorescent phospholipids from BMVs at low temperature or use the dithionite reduction assay (McIntyre & Sleight, 1991; Huijbregts et al., 1996) under conditions where we could guarantee that dithionite did not permeate the BMV membrane bilayer. Another potential assay based on the conversion of exogenously added phosphatidylserine to phosphatidylethanolamine by the cytoplasmically located enzyme phosphatidylserine decarboxylase (Huijbregts et al., 1996) did not work owing to essentially immeasurable decarboxylase activity, a consequence of the low level of this enzyme in *B. megaterium* (Langley et al., 1979).

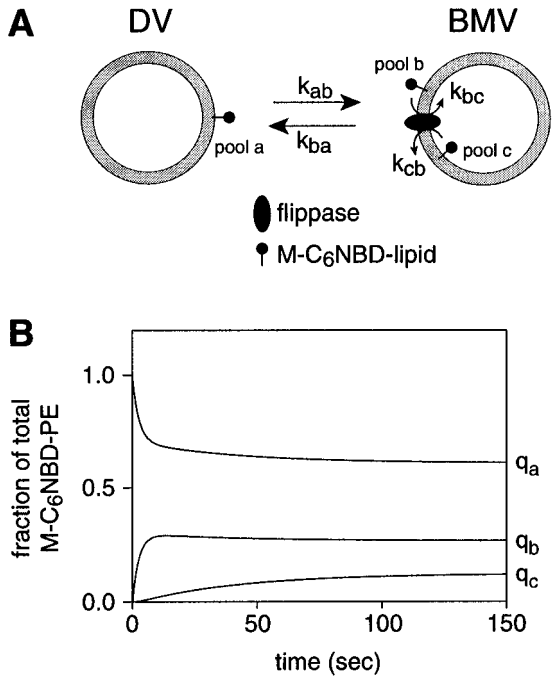

FIGURE 7: Evolution of the DV and BMV pools of M-NBD-PE as a function of time. Experimentally measured rate constants (k_1 and k_2), amplitudes (A_1 and A_2), and relative fluorescence yields of M-NBD-PE in DVs and BMVs were used to model the time evolution of the three membrane-bound pools of M-NBD-PE in a transport assay using BMV acceptors. The graph shows the dynamic behavior of the M-NBD-PE pools in DVs (q_a) and BMVs (BMV outer leaflet pool, q_b; BMV inner leaflet pool, q_c). The pools are represented as fractions of the total transportable M-NBD-PE in the assay. Thus at time = 0 s, $q_a = 1$, $q_b = 0$, and $q_c = 0$. The rate constants and amplitudes used for the model were obtained from an assay performed with DV suspension of 0.4 nmol of lipid phosphorus, and 10 μL BMV suspension (= 6.1 nmol lipid phosphorus) in a total volume of 2 mL. For the plot shown, the rate parameters (see Experimental Procedures for details) determined from the model were $k_{ab} = 0.112\ s^{-1}$, $k_{ba} = 0.242\ s^{-1}$, $k_{bc} = 0.008\ s^{-1}$, $k_{cb} = 0.021\ s^{-1}$. The ratio of the outer to inner leaflet pool BMV pools at equilibrium was $k_{cb}/k_{bc} = 2.6$, *i.e.*, 73%−27% outside−inside.

Mathematical Modeling of M-NBD-Phospholipid Transport: Estimation of Kinetic Parameters and the Transbilayer Distribution of M-NBD-PE in BMVs

In order to gain insight into the time evolution of the pools of M-NBD-phospholipid in the transport assay, and also to estimate the transbilayer distribution of the lipid probe in the BMV membrane, we modeled our data using a three-pool kinetic model (Shipley & Clark, 1972) (Figure 7A). Using experimentally determined values for the rate constants k_1 and k_2, the amplitudes A_1 and A_2, and the relative fluorescence yields of the M-NBD-phospholipid in donor vesicles and BMVs, we obtained kinetic parameters that allowed us to describe the time evolution of the tracer pool in the DVs (q_a), and the outer (q_b) and inner (q_c) leaflets of the BMVs; the model ignores the pool of tracer in the aqueous phase by assuming that this pool reaches equilibrium rapidly.

A graph of the time dependence of the three pools (represented as fractions of the total transportable M-NBD-phospholipid) is shown in Figure 7B. The data were obtained from a measurement of M-NBD-PE transport between DVs and BMVs. As seen in Figure 7B, the pool of M-NBD-PE in the DVs is rapidly depleted, reaching a plateau in about 25 s. In contrast, the M-NBD-PE pool in the BMV outer leaflet grows rapidly and then flattens out as transbilayer movement occurs to populate the BMV inner leaflet.

Transport equilibrium in the three-pool system is reached in about 100 s (Figure 7B). At this point, for the data shown in Figure 7B, the M-NBD-PE pools in the outer and inner leaflet of the BMVs correspond to ~0.28 and ~0.11, respectively (the total transportable M-NBD-PE is set at 1.0), *i.e.*, a transbilayer distribution of 73% outside and 27% inside. These numbers vary somewhat, ranging from 73%−27% outside−inside to 62%−38% outside−inside depending on the experiment. The results are in general agreement with the pool size estimate (60%−40% outside−inside) obtained via cobalt quenching (Figure 6B, BMV trace). Experimental data for M-NBD-PG were also transformed using the model, and gave similar results, with 64%−36% to 56%−44% outside−inside. Considering the difference in area between the outer and inner leaflet [≈60% of total phospholipids in the outer leaflet of vesicle of this size, *i.e.*, ~280 nm diameter (Konings et al., 1973)] the outside−inside distributions of M-NBD-PE and M-NBD-PG that we obtained suggest that the fluorescent analogs equilibrate at an equal concentration in the outer and inner leaflets of the BMV membrane. These results contrast with the asymmetric distribution of endogenous PE and PG in the *B. megaterium* membrane described by Rothman and Kennedy (1977b). These authors measured the transbilayer distribution of PE in intact bacteria as roughly 30% outside−70% inside and inferred that PG was distributed with the opposite asymmetry, *i.e.*, 70% outside−30% inside. Our result that M-NBD-PE and M-NBD-PG are roughly equally distributed in both leaflets of the BMVs may be plausibly explained by suggesting that transbilayer asymmetry may be determined by membrane-associated proteins or surface constraints that are absent in the BMV preparations that we use. Attempts to use these methods to study transmembrane movement and phospholipid asymmetry in intact bacteria were not particularly informative as the intermembrane transfer event was slower than in BMVs, possibly as a result of interference from the bacterial wall, making it difficult to distinguish subsequent transbilayer movement.

Summary

In summary, we have developed an on-line fluorescence assay to monitor phospholipid flippase activity in vesicle preparations. The assay is based on work by Nichols and Pagano (1981, 1982; Zhang & Nichols, 1994) and offers a number of advantages (such as greatly improved time resolution) over assays currently in use to monitor flippase activity. We used the assay to study the characteristics of phospholipid flipping in *B. megaterium* membrane vesicles, a biogenic membrane system endowed with the ability to promote transbilayer movement of phospholipids to a biologically relevant rate (Rothman & Kennedy, 1977; Langley & Kennedy, 1979; Menon, 1995). We show that the "flippase" activity in *B. megaterium* vesicles results in rapid, nonselective transbilayer movement of fluorescent phospholipid analogs. The rate of transbilayer movement is reduced by proteolysis of the vesicles, suggesting that transbilayer transport is protein-mediated. The availability of a biochemically tractable assay for transbilayer movement

of phospholipids in bacterial systems should provide fresh impetus for analyses of the transport mechanism.

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