Transbilayer Movement of Fluorescent Phospholipid Analogues in the Cytoplasmic Membrane of *Escherichia coli*†

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ABSTRACT: We investigated the transmembrane movement of fluorescent labeled phospholipids in inverted inner membrane vesicles (IIMV) of *Escherichia coli* (*E. coli*) wild-type strain (MG1655), as well as in proteoliposomes reconstituted from detergent extracts of the IIMV. The transbilayer movement of 1-myristoyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphoethanolamine (M-C6-NBD-PE) and -phosphocholine (M-C6-NBD-PC) was measured by a fluorescence stopped-flow back-exchange assay. Both analogues were rapidly translocated across the IIMV membrane, with half-times of <1 min (outward movement) and ∼3 min (inward movement). No flip-flop was detected in protein-free liposomes, but in IIMV-derived proteoliposomes flip-flop of M-C6-NBD-PE occurred similarly to IIMV and could be largely eliminated by proteinase K treatment.

In Gram-negative bacteria, like *E. coli*, newly synthesized phospholipids have to move from the cytoplasmic leaflet of the inner membrane, where their synthesis takes place, to the periplasmic leaflet of the inner membrane and to the inner leaflet of the outer membrane. Even though both processes have been studied in in vivo and in vitro systems [for a recent review, see (*1*)], the mechanism by which phospholipids flip across the inner membrane is still unknown.

Because transbilayer translocation of phospholipids is very slow (hours up to days) in the bulk lipid phase, it is generally assumed that protein(s) are required to facilitate the translocation process. The first report on transbilayer movement of phospholipids in bacteria came from Rothman and Kennedy (*2*), who concluded that the translocation of newly synthesized phosphatidylethanolamine (PE)[1] from the inner to the outer leaflet in *Bacilli* occurred with a half-time of 1.5−3 min at 37 °C. A more recent study (*3*) showed that short-chain fluorescent labeled phospholipid analogues translocated rapidly across *Bacillus megaterium* membrane vesicles with a half-time of ∼30 s at 37 °C. This process was demonstrated to be protease-sensitive but not headgroup-specific.

A study published last year (*4*) described the reconstitution of transport-competent proteoliposomes from detergent-solubilized *Bacillus subtilis* membranes. The proteoliposomes were shown to be capable of transporting a short-chain analogue of phosphatidylcholine (PC) as well as the more natural lipid dipalmitoyl-PC. Although the transport assays used lacked the time resolution to measure a translocation rate, fractionation of the detergent extract prior to reconstitution indicated a clear requirement for specific proteins to facilitate PC flip-flop.

In this study, we extend the progress that has been made recently, by analyzing phospholipid flip-flop in native and reconstituted *E. coli* inner membranes. Previous work (*5*, *6*) on phospholipid flip-flop in *E. coli* inner membranes tested transport of phospholipid analogues as well as endogenous phospholipids. Although a flip-flop rate could not be determined in these studies, it was concluded that, as with *Bacillus*, flip-flop in *E. coli* inverted inner membrane vesicles (IIMV) is rapid and occurs independently of metabolic energy. However, these earlier studies could not demonstrate a requirement for protein in the translocation process. It was also not known whether the rapid flip-flop seen in IIMV could be reconstituted in proteoliposomes derived from a detergent extract of IIMV.

Here we have measured the transbilayer movement and distribution of fluorescent, 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled, short-chain phospholipid analogues in *E. coli* IIMV and in proteoliposomes reconstituted from detergent extracts of IIMV. We took advantage of the high time resolution of the stopped-flow technique (death time ∼10 ms) in combination with BSA extraction (the classical "back exchange" assay) to monitoring the transbilayer movement

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[1] Abbreviations: IIMV, inverted inner membrane vesicles; BSA, bovine serum albumin; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PC, phosphatidylcholine; PE, phosphatidylethanolamine; M-C6-NBD-PC, 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphocholine; M-C6-NBD-PE, 1-myristoyl-2-[6-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl]-*sn*-glycero-3-phosphoethanolamine; TLC, thin-layer chromatography.

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of the analogues (*7*). Fits of the data to a three-compartment model revealed half-times for the inward and the outward translocation of the phospholipid analogues in the range 0.5−3 min. Importantly, the experiments clearly demonstrated protease-sensitivity of the transport process and indicated that translocation in proteoliposomes can be eliminated by proteinase K treatment. These data provide the first time-resolved measurements of the flip-flop of phospholipid analogues in *E. coli* and demonstrate a clear requirement for protein(s) in the transport process. Furthermore, they show that flippase activity can be reconstituted in proteoliposomes without significant impairment of its activity, providing the basis for identification of the protein.

MATERIALS AND METHODS

Chemicals. M-C6-NBD-PE and M-C6-NBD-PC were obtained from Avanti Polar Lipids (Alabaster, AL). Egg PC and BSA were from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Triton X-100 was purchased from Fluka Chemie AG (Buchs, Switzerland). SM-2 Bio-Beads were obtained from Bio-Rad Laboratories.

Preparation of Inverted Inner Membrane Vesicles. Inner membrane vesicles were isolated from the *E. coli* wild-type strain MG1655. Cell growth and membrane isolation were done as described by Huijbregts et al. (*5*). Briefly, cells were grown at 37 °C in Luria Broth (LB). Overnight cultures were diluted into 3 L of LB and grown to an OD_{660} of 0.7−0.8 (late log phase). The cells were harvested by centrifugation, washed in ice-cold physiological buffer, and resuspended in 40 mL of buffer S (50 mM triethanolamine−HOAc, pH 7.5, 250 mM sucrose, 1 mM EDTA). The suspension was supplemented with 1 mM DTT and 0.375 mM PMSF. Cells were broken by 2−3 passages in a French press at a cell pressure of 8000 psi. Additional DTT (1 mM) was added after each passage. Cell debris were removed by centrifugation (2× for 10 min at 6000*g* in a 45Ti Beckman rotor). The outer membrane fraction was pelleted by centrifuging at 165000*g* for 1 min in an SW28 Beckman rotor. The supernatant was then centrifuged at 165000*g* for 90 min in an SW28 Beckman rotor to pellet the crude inverted inner membrane vesicles. The crude IIMV pellet was resuspended in 2 mL of buffer S and layered on top of a discontinuous sucrose gradient according to Osborn et al. (*8*) and centrifuged for 16−18 h in an SW40 Beckman rotor at 112000*g*. Three bands were visible. The two light bands [corresponding to the purified IIMV, (*8*)] were collected and washed in buffer S without sucrose at 165000*g* for 90 min (SW28 rotor, Beckman). Vesicles were resuspended in HEPES (20 mM, 100 mM NaCl, pH 7.5) using a dounce homogenizer, quickly frozen in liquid nitrogen, and stored at −80 °C. IIMV suspensions were thawed immediately before use.

IIMV isolated as described above typically contained ∼3 mg of protein/μmol of phospholipid. It has been already shown previously (*5*) that IIMV prepared by this method are sealed and primarily inside-out. The contamination of isolated IIMV with outer membrane fractions is neglible as verified by a phospholipase assay (see below).

Reconstitution Procedure. IIMV were solubilized and reconstituted according to the method described by Menon et al. (*10*) and Hrafnsdóttir and Menon (*4*). To this end, the vesicle suspension was mixed with an equal amount of buffer R2 [20 mM HEPES, pH 7.5, 200 mM NaCl, 2% (w/v) Triton X-100]. The mixture was incubated on ice for 30 min and subsequently centrifuged in a 70.1 Ti Beckman rotor at 175000*g* for 45 min. The supernatant was carefully collected and stored on ice before further reconstitution steps. The Triton extract was added to a solution of eggPC in buffer R1 [10 mM HEPES, pH 7.5, 100 mM NaCl, 1% (w/v) Triton X-100] with a final phospholipid concentration (after addition of the Triton extract) of 4.5 μmol/mL. In some cases, the fluorescent phospholipid analogue M-C6-NBD-PE was added with a final concentration of 2 mol % of total phospholipid content. For detergent removal, 100 mg/mL (wet weight) SM-2 Bio-Beads were added and gently rocked for 3 h at room temperature. Subsequently, additional Bio-Beads were added (200 mg/mL wet weight), rocking was continued for an additional 2 h at room temperature, and the mixture was transferred to 4 °C and gently rocked for a further 14−18 h. The resulting turbid suspension was separated from the Bio-Beads and centrifuged at 175000*g*, 45 min, 4 °C (70.1 Ti Beckman rotor). The resulting pellet was resuspended in 2 mL of buffer (10 mM HEPES, pH 7.5, 100 mM NaCl), centrifuged again at 175000*g* for 45 min, 4 °C, resuspended in the same buffer, and homogenized with a dounce homogenizer on ice.

Enzyme Assays. To test the degree of contamination of the IIMV preparation with outer membrane, we measured the activity of phospholipase A2, an outer membrane marker. To this end, 5 nmol of headgroup-labeled N-Rh-PE was diluted in 1 mL of chloroform. Then 11 μL of this suspension was dried under a gentle stream of nitrogen, and 5 μL of 50 mM Tris (pH 8), 20 mM calcium chloride, 0.2% Triton X-100 and 5 μL of the respective fraction were added. The incubation mixture was allowed to react at 37 °C for 1 h. After incubation, 22 μL of chloroform/methanol (1:1) was added, vortexed, and centrifuged at 1000*g* for 2 min. Thin-layer chromatography (TLC) of the lower phase was carried out with chloroform/methanol/water (65:25:4) on silica 60 plates. The spots were analyzed using a VD 40 Hitachi 3-Chip analyzing system (Desaga GmbH, Wiesloch, Germany). Phospholipase A2 activity was detected by spots corresponding to the lyso derivative of N-Rh-PE. The analysis was performed with the standard accessory software (ProViDoc 3.04). While for fractions of the outer membrane a high degree of phospholipase A2 activity was detected, any appearance of lyso-N-Rh-PE in the fraction of IIMV was below the detection level.

Stopped-Flow Measurements. All stopped-flow measurements were performed at room temperature. The time-dependent back-exchange of NBD-labeled phospholipids was monitored by mixing the labeled IIMV with 2% (w/v) BSA (final concentration) in 10 mM HEPES buffer (pH 7.5) using a stopped-flow accessory (RX 1000, Applied Photophysics, Leatherhead, U.K.) linked to an Aminco Bowman Series 2 spectrofluorometer (SLM Instruments). The quantum yield of NBD analogues bound to BSA is lower compared to that of analogues incorporated into membranes [see also (*7*) and Figure 4]. Thus, extraction of analogues from membranes by BSA can be followed by the decrease of fluorescence intensity. Fluorescence was recorded for 300 s at a time resolution of 0.2 or 0.5 s, excitation wavelength $\lambda_{ex} = 477$ nm, emission wavelength $\lambda_{em} = 540$ nm, slit widths = 4 nm. For each preparation, five or more kinetic traces were

recorded and averaged for kinetic analysis (see below). However, scattering of (nonlabeled) IIMV and IIMV-derived proteoliposomes was significant, and it was necessary to correct the traces to compensate for the light scattering contribution to the amplitude of the signal. Despite this correction, the amplitudes of the kinetics are afflicted with a slight uncertainty.

Kinetic Analysis. The experimental data were fitted to a theoretical time course using a three-compartment model (Figure 1). This kinetic model describes transbilayer movement as well as the transfer of phospholipid analogues between the outer leaflet of the membrane vesicle and BSA. The outward and inward movements of phospholipid analogues are described by the rate constants k_{+1} and k_{-1}, respectively. The movement of the analogues from the IIMV to BSA is characterized by the rate constant k_{+2} (extraction of the analogues by BSA) and k_{-2} for the movement of analogues back from BSA to the vesicle membrane. We note that due to the excess of BSA used, the exchange process described by k_{-2} did not contribute to the kinetics, and the values for this time constant were very small (typically 10^{-12} s^{-1}) as expected.

$[PL_o]$ and $[PL_i]$ are the concentrations of analogue in the outer and inner leaflet of the IIMV. At the time of BSA addition ($t = 0$ s), the transmembrane distribution is at steady state, i.e.:

$$\frac{[PL_o]_{t=0}}{[PL_i]_{t=0}} = \frac{k_{+1}}{k_{-1}} \quad (1)$$

The concentration of analogue transferred to BSA ($[PL_{tr}]$) is taken to be zero at the time of addition.

The model is represented by the following system of differential equations:

$$\frac{d[PL_i]}{dt} = -k_{+1}[PL_i] + k_{-1}[PL_o] \quad (2)$$

$$\frac{d[PL_o]}{dt} = k_{+1}[PL_i] - (k_{-1} + k_{+2})[PL_o] + k_{-2}[PL_{tr}] \quad (3)$$

$$\frac{d[PL_{tr}]}{dt} = k_{+2}[PL_o] - k_{-2}[PL_{tr}] \quad (4)$$

$$[PL_{tr}] = C - [PL_i] - [PL_o] \quad (5)$$

$$C = [PL_i]_{t=0} + [PL_o]_{t=0} + [PL_{tr}]_{t=0} \quad (6)$$

For further details, see Marx et al. (*7*). Fitting was performed by least-squares minimization.

Proteinase K Treatment. Aliquots of labeled proteoliposomes were incubated for 30 and 60 min at room temperature with 1 mg of proteinase K/mL. Proteolysis was terminated by adding 3 mM PMSF, and the stopped-flow assay was performed as described above. As a control, an aliquot of labeled vesicle suspension was treated with buffer only, incubated for the indicated time, and then supplemented with 3 mM PMSF.

Miscellaneous. Triton X-100 absorbance was used to check the removal of detergent during reconstitution as described by Hrafnsdóttir and Menon (*4*). The phospholipid content of IIMV and reconstituted vesicles was determined as described by Hrafnsdóttir and Menon (*4*) after lipid isolation according to Bligh and Dyer (*9*). The stability of fluorescent analogues during labeling and in the time course of subsequent experiments was verified by TLC (data not shown).

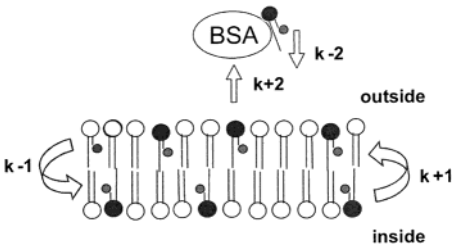

FIGURE 1: Kinetic model describing transfer of NBD-labeled phospholipid analogues between biological membranes and BSA. The outward and inward transbilayer movements of analogues are characterized by k_{+1} and k_{-1}, respectively. The rate constant of the extraction of phospholipid analogues by BSA is represented by k_{+2}, and k_{-2} describes the backward movement of analogues from BSA into solution or membrane.

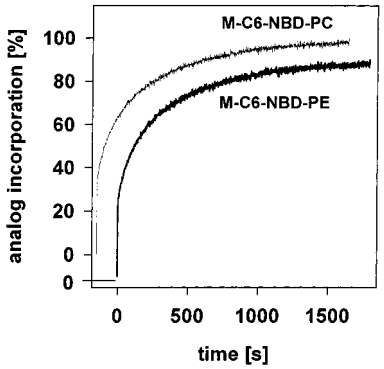

FIGURE 2: Kinetics of incorporation of NBD-labeled phospholipid analogues into IIMV membranes. 2 mol % M-C6-NBD-PE or M-C6-NBD-PC, according to the total lipid content of the IIMV, was added to IIMV (12.5 μM final phospholipid concentration) in HEPES buffer, and the kinetics of membrane incorporation of the analogues were followed. The kinetics for PE analogues are displaced in the figure for clarity. At time point zero, the label was added to the IIMV suspension. The value of 100% corresponds to incorporation of all fluorescent labeled analogues. All experiments were performed at room temperature. Note that these experiments were performed in a regular quartz cuvette without employing stopped-flow equipment. Therefore, due to the lower time resolution, the initial fluorescence increase upon mixing of IIMV with lipid analogues could not be adequately resolved.

RESULTS

We first analyzed the incorporation of M-C6-NBD-PE and M-C6-NBD-PC into IIMV by cuvette experiments (Figure 2). In aqueous solution, the NBD-labeled phospholipid analogues are organized mainly in micelles where their fluorescence is self-quenched. Upon adding acceptor membranes, such as IIMV, the fluorescence increases since the lipid molecules spontaneously incorporate into membranes and thus micelles are dissolved. Incorporation of lipids into membranes can therefore be directly monitored by the fluorescence increase. An aliquot of IIMV was mixed with HEPES buffer containing fluorescent analogues (2 mol % of total phospholipid content), and the resulting increase of fluorescence intensity was monitored.

As evident from the kinetics in Figure 2, an initial rapid phase of intercalation of analogues into IIMV was observed. After 10 min, about 90% of the analogues were incorporated.

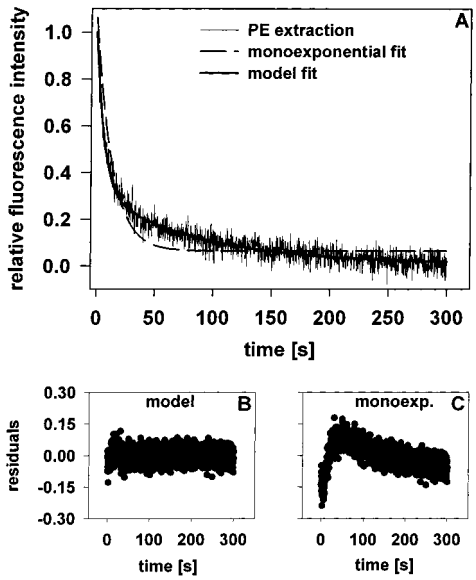

FIGURE 3: Kinetics of extraction of M-C6-NBD-PE from IIMV by BSA. 25 μL of IIMV (2.89 mg of protein/μmol of phospholipid) was incubated with 2 mL of a buffered suspension of M-C6-NBD-PE (2 mol % of the lipid content) for 30 min at room temperature. Subsequently, the labeled IIMV were rapidly mixed with an equal volume of 4% (w/v) BSA in a stopped-flow accessory, and the fluorescence decay was recorded with a time resolution of 0.2 s. The kinetics were normalized as follows: the initial fluorescence intensity (before BSA extraction) was set to 1, and the intensity after 300 s to 0. The curve represents the average of five measurements (A). The solid gray line represents the fit obtained by fitting our data to the three-compartment model. The dashed line was obtained by fitting the data to a monoexponential function. The residuals of the model fit (B) and the monoexponential fit (C) are depicted.

However, the final plateau was reached within 30 min. Essentially all of the PE and PC analogues were incorporated into IIMV. This was verified by the fact that no further fluorescence increase was observed when an additional aliquot of IIMV was added to the suspension (data not shown).

For the measurement of the transbilayer movement of M-C6-NBD-PE and M-C6-NBD-PC across the IIMV membranes, we used stopped-flow methods taking advantage of the fact that the lipid analogues can be extracted from the membrane by BSA ("back exchange") and that the quantum yield of analogues bound to BSA is different from that of membrane-incorporated analogues. We used cuvette experiments to verify that the quantum yield of analogues bound to BSA is less (about 55%) than that found in membranes (see Figure 4). After labeling of IIMV with 2 mol % fluorescent analogues for 30 min at room temperature, vesicles were rapidly mixed with 2% (w/v) BSA solution (final concentration) by stopped-flow (dead time of mixing ~10 ms). The time-dependent decrease of fluorescence resulting from back-exchange of analogues by BSA was monitored. As seen in Figure 3 (only shown for M-C6-NBD-PE), the fluorescence emission intensity was found to decay in two distinct phases. After 300 s, no change of fluorescence was observed, suggesting that all phospholipid analogues were extracted and bound to BSA (see below). Therefore, the kinetics were normalized as follows: the initial fluorescence intensity (before BSA extraction) was set to 1, and the intensity after 300 s to 0.

Table 1: Half-Times of Transbilayer Movement and Extraction of M-C6-NBD-PE and M-C6-NBD-PC across IIMV Membranes and Proteoliposomes Reconstituted from Detergent Extract[a]

analogue	outward movement (s)	inward movement (s)	extraction (s)	$[PL_l]_{t=0}$ (%)
IIMV				
M-C6-NBD-PE ($n = 2$)	58	160	4.6	26.6
	48	210	2.5	18.6
M-C6-NBD-PC ($n = 2$)	17	100	1.1	14.7
	63	242	2.7	20.7
proteoliposomes				
M-C6-NBD-PE ($n = 4$)	29 ± 4	61 ± 11	6.4 ± 1.3	32.6 ± 3.7

[a] Half-times of the outward and inward movement were derived from k_{+1} and k_{-1}, respectively (see Figure 1). $[PL_l]_{t=0}$ corresponds to the relative amount of analogues on the lumenal site of vesicles at $t = 0$. For IIMV, the lumenal site corresponds to the periplasmic leaflet. Parameters were obtained from the experimental curves (see Figure 3A and Figure 6) according to the model described under Materials and Methods. The average and standard deviation are given. *n* refers to the number of independent experiments. For each experiment, at minimum five single kinetics were averaged and subsequently analyzed.

We propose that the fast initial decrease of fluorescence intensity reflects the extraction of phospholipid analogues localized in the outer leaflet of the vesicles. We further propose that the second slower phase is caused by extraction of M-C6-NBD-PE translocated from the cytoplasmic leaflet to the outer leaflet. To determine the characteristic half-times of the two phases, the data were fitted as described under Materials and Methods (see Figure 3). Additionally, a monoexponential fit was performed (Figure 3A, dashed line) to compare the model-based data with a simple virtual one-phase process. The residuals (Figure 3C) clearly show that a monoexponential function does not fit the recorded data. However, the data could be well fitted by a biphasic process (Figure 3A, solid line, and Figure 3B). On the basis of the three-compartment model depicted in Figure 1, the half-times of flip-flop and the extraction of analogues as well as their transbilayer distribution were estimated (Table 1). The transbilayer dynamics and distribution of M-C6-NBD-PE and M-C6-NBD-PC were found to be almost identical. While data in Table 1 refer to measurements of IIMV samples labeled for 30 min with NBD-lipids, we found essentially the same results when performing stopped-flow measurements on IIMV preparations labeled for 90 min. This indicates that after 30 min of labeling the analogues are already equilibrated between the two leaflets of the bilayer, consistent with a rapid flip-flop of the phospholipid analogues.

To verify whether all analogues were extracted by BSA, we labeled an aliquot of IIMV with 2 mol % M-C6-NBD-PE or M-C6-NBD-PC, then incubated the vesicles with 2% (w/v) BSA for 300 s at room temperature, and measured the resulting NBD fluorescence. The fluorescence intensity was essentially the same as that found when an equal amount of analogues in aqueous suspension was incubated with 2% (w/v) BSA (Figure 4B,C) and about 50% of that seen when the analogues were all membrane-integrated (compare Figure 4B with Figure 4A). Our data clearly show that all analogues were completely extracted by BSA after 300 s. Additionally,

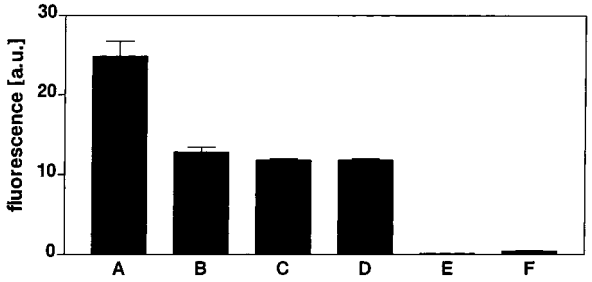

FIGURE 4: Complete extraction of phospholipid analogues from IIMV by BSA. IIMV (60 μM final phospholipid concentration) were labeled with 2 mol % M-C6-NBD-PE for 30 min (A); subsequently, the analogues were extracted by 2% (w/v) BSA in 10 mM HEPES for 300 s (B). Longer incubation (>300 s) did not change the fluorescence intensity. (C) BSA [2% (w/v)]-bound M-C6-NBD-PE (2 mol % of total phospholipid content of IIMV) in the absence of IIMV. (D) An aliquot of IIMV was added to BSA-bound M-C6-NBD-PE (2 mol % of total phospholipid content of IIMV). (E) IIMV [equal to the amount used in (A) and (D)] in 2 mL of HEPES buffer. (F) 2% (w/v) BSA in 2 mL of HEPES buffer. The error bars indicate the standard deviation of two experiments. The experiment was performed at room temperature.

when an aliquot of IIMV was added to the sample containing the BSA-bound analogues, no changes occurred due to the presence of IIMV (Figure 4D), consistent with the proposal that k_{-2} (transfer of analogues from BSA to membranes) contributes very little to our analysis (Figure 1).

To analyze the mechanism of phospholipid flip-flop further, we reconstituted a series of proteoliposomes from IIMV containing different amounts of protein extract and 2 mol % M-C6-NBD-PE (see Materials and Methods). We estimated that by this procedure ~50−70% of the protein and ~60−85% of phospholipid from the reconstitution mixture were recovered in the resulting proteoliposomes, while greater than 99% of the starting Triton X-100 was removed. The proteoliposomes were rapidly mixed with a BSA solution by stopped-flow, and the time-dependent decrease of fluorescence caused by back-exchange of analogues by BSA was monitored. We studied only M-C6-NBD-PE, not M-C6-NBD-PC, since we did not find significant differences between the transbilayer movement of the two analogues in IIMV [see also (*3*) for an equivalent result using *Bacillus* membranes]. As with IIMV, we found a biphasic decline of the fluorescence intensity on adding BSA to the M-C6-NBD-PE-labeled proteoliposomes—an initial rapid decline followed by a second phase of slow fluorescence decrease. The kinetics of fluorescence decrease were independent of protein content (see Discussion). At high protein content (180 μg/mL, Figure 5), we observed that the fluorescence decreased finally to about 65% of the initial value. Based on quantum yields, this indicates that most of the analogues (≥90%) are extracted from the proteoliposomes to BSA. At lower protein contents, removal of analogues was not complete, indicating that a fraction of the proteoliposomes lacked the putative flippase protein (Figure 5). For vesicles reconstituted in the absence of protein extract but in the presence of Triton X-100, we found only the rapid initial phase of fluorescence decline but no further (slow) decrease (Figure 5). The fluorescence in the liposome sample reached a final plateau between 80 and 85%, consistent with the removal of about 50% of the analogues by BSA. These results are consistent with our interpretation that the initial phase corresponds to extraction of the analogues from the outer leaflet while the later "slow" phase corresponds to the transbilayer movement. For proteoliposomes with almost complete analogue extraction (≥90%) as deduced from the quantum yield, we fitted the fluorescence traces to the model. From fitting of the experimental data to the model, we determined that the transbilayer movement of NBD-phospholipids in proteoliposomes (Figure 6) was very similar to that seen in IIMV. The respective rate constants were of the same order as those found for IIMV (Table 1). Extraction kinetics of protein-free liposomes could be fitted to a monoexponential function (data not shown). We note that amplitudes for extraction present only rough estimates due to significant scattering (see Materials and Methods).

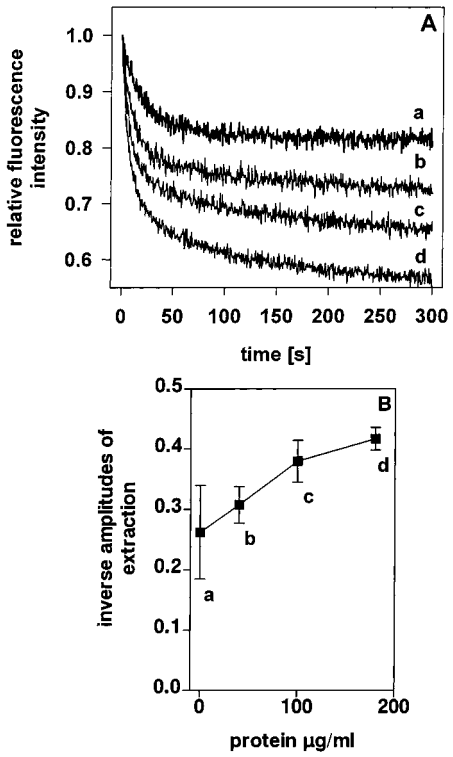

FIGURE 5: Effect of protein concentration on flip-flop of and extraction of M-C6-NBD-PE. (A) The proteoliposomes were prepared with increasing amounts of the Triton extract and labeled with M-C6-NBD-PE during the reconstitution procedure. Subsequently, the labeled proteoliposomes made from IIMV were analyzed by the stopped-flow assay as described before. The kinetics represent the mean of 8 records corresponding to pure liposomes (trace a), 6 records to 40 μg of protein/mL (trace b), 8 records to 100 μg of protein/mL (trace c), and 9 records to 180 μg of protein/mL (trace d). For all samples, the phospholipid concentration was the same (see Materials and Methods). Setting all the extractable analogues to 100%, the relative contribution of the fast component (analogues at $t = 0$ on the outer leaflet) decreases from 100% (curve a) to 66% (curve d), while the relative contribution of the slow component (analogues in the inner leaflet at $t = 0$, which become accessible to BSA after redistribution to the outer leaflet) increases from 0% to 34%. For fitting of curves and half-times of transbilayer movement of analogues, see Figure 6 and Table 1. (B) The final amplitudes of fluorescence decrease [see (A)] of two independent experiments with increasing amounts of proteins are shown. The error bars depict the standard deviation. The letters a−d next to each data point correspond to the traces (for one of the two experiments) in panel A.

Taken together, these results strongly indicate that the rapid transbilayer movement of M-C6-NBD-PE in reconstituted vesicles is protein-dependent. This conclusion is further supported by the influence of proteinase K pretreatment of

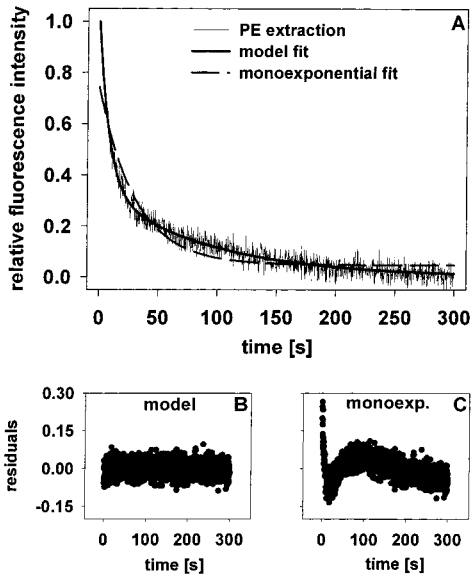

FIGURE 6: Stopped-flow kinetics of extraction of M-C6-NBD-PE from reconstituted IIMV by BSA. IIMV-derived proteoliposomes were labeled with 2 mol % of the fluorescent phospholipid analogues during the reconstitution as described under Materials and Methods. Equal volumes of proteoliposomes and 4% (w/v) BSA were mixed in the stopped-flow apparatus, and subsequently the fluorescence decrease was recorded at room temperature (A). The curve represents the average of five separate kinetic traces. Traces were corrected for scattering (see Materials and Methods). The solid lines represent the fit of the experimental data to the model shown in Figure 1. The dashed line was obtained by a monoexponential fit of the data. The residuals for a fit of the data to the model (B) and of a monoexponential fit (C) of the extraction kinetics are displayed. The half-times obtained from independent preparations are summarized in Table 1.

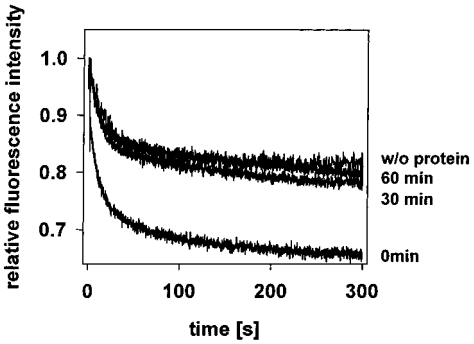

FIGURE 7: Kinetics of the extraction of M-C6-NBD-PE from reconstituted IIMV by BSA prior to and after proteinase K treatment. Reconstituted IIMV-derived proteoliposomes were labeled with 2 mol % M-C6-NBD-PE during reconstitution as described before, and a stock was treated with proteinase K (1 mg/mL) for the indicated time. An aliquot was removed from the stock and incubated with 3 mM PMSF (final) for 5 min prior to analysis by stopped-flow back-exchange. Traces were corrected for scattering (see Materials and Methods). For comparison, the extraction kinetics measured with pure liposomes (without protein) are shown.

proteoliposomes on analogue extraction. The amount of extractable analogues decreased dramatically after a 30 or 60 min pretreatment with proteinase K (Figure 7) as revealed by comparison with the kinetics of pure liposomes. This indicates that the proteolysis eliminated flippase activity in a large fraction of the proteoliposome population. Notably, despite aggressive treatment with proteinase K (incubation for 60 min), we could not completely eliminate the pool of transport-active vesicles. The half-times of transbilayer movement of analogues in those vesicles were of the order of those given for proteoliposomes in Table 1.

DISCUSSION

In this study, we investigated the transbilayer movement of fluorescent phospholipid analogues in IIMV derived from *E. coli* wild-type strain MG1655, as well as in proteoliposomes generated from detergent extracts of IIMV. To do this, we combined the commonly used BSA back-exchange procedure with stopped-flow methods. This combination enabled us to record fluorescence changes that occurred during the extraction of fluorescent-labeled phospholipid analogues from IIMV and proteoliposomes directly and with high time resolution. Subsequently, the data were analyzed via a three-compartment model (*7*) that allowed us to deduce the rate constants (half-times) of transbilayer movement and calculate the distribution of phospholipid analogues on the two sides of the bilayer. The applicability of this stopped-flow back-exchange approach depends on the quantitative relation between the rate constants (or respective half-times) for extraction of analogues by BSA and the rate constants for the transbilayer movement of analogues. The latter can be measured by our approach only if the extraction step is significantly faster in comparison to the transbilayer movement of phospholipid analogues. We found that the analogues were rapidly extracted from the outer leaflet of membrane vesicles (IIMV or proteoliposomes) with a half-time of <7 s, very similar to that observed for microsomes from rat liver cells (*7*). For IIMV and proteoliposomes, but not for liposomes, a second slower decrease of fluorescence was recorded. We attribute this second phase to the transbilayer translocation of the analogues with half-times between 40 and 185 s as revealed from the analysis of extraction kinetics. This indicates that the process of phospholipid analogue flip-flop is much slower than that of analogue extraction by BSA.

We found that the movement of both analogues to the periplasmic leaflet of IIMV is about 3−4 times slower than that to the cytoplasmic layer. Our data showed that only ∼22% of the PE analogue and 17% of the PC analogue are located in the periplasmic leaflet of the vesicle membrane. This transbilayer distribution of analogues is in accordance with previous observations of the distribution of NBD-PE in the inner membrane of *E. coli* (*5*). However, recently it was shown that PE is preferentially (65%) redistributed to the periplasmic leaflet (*6*). The discrepancy might be related to the chemical nature of analogues, causing them to behave differently from their endogenous counterparts, and/or to a specific transbilayer distribution of newly synthesized PE. Moreover, it is not known how the isolation procedure of IIMV affects the transbilayer distribution of phospholipids. Nevertheless, the transbilayer movement of analogues mimics adequately that of newly synthesized PE. Although the translocation half-times of the latter could not be resolved in a previous study, it was estimated that they are of the order of 1 min or less (*6*).

Here we set up a reconstitution assay to investigate the transbilayer movement of fluorescent phospholipid analogues in more detail. We created vesicle populations with increasing protein-to-phospholipid ratios and tested these preparations in the back-extraction/stopped-flow assay. We found that with increasing amounts of protein the fluorescence

amplitude decreased (Figure 5), reflecting the fact that an increasing amount of vesicles are able to transport the phospholipid analogues across the membrane. As displayed in Figure 5B, the transport maximum seen with vesicles of high protein content (180 μg/mL) was close to the plateau value expected if all vesicles were transport-competent. Thus, in a vesicle population prepared with ~180 μg/mL protein, most vesicles are equipped with a putative flippase. As deduced from the rate constants (see legend to Figure 5), the kinetics of transbilayer movement of M-C6-NBD-PE in proteoliposomes prepared in the range 0−180 μg/mL (Figure 5B) were essentially independent of protein content. We suggest that in this range of protein content the proteoliposome population contains vesicles with at most one transporter (flippase), with some vesicles lacking transport activity. We anticipate that only once the protein content of the proteoliposome preparation exceeds 180 μg/mL will rate increases become evident.

As shown in Table 1, the transbilayer movement of the PE analogue in proteoliposomes is very similar to its movement in IIMV. This shows that the flip-flop in IIMV is protein-dependent and can be reconstituted without significant impairment. The difference between the inward and outward movement was smaller than that found for IIMV. This implies that the activity of a putative flippase has a preference toward the cytoplasmic leaflet of IIMV. Very likely, for proteoliposomes one would expect that the specific transbilayer orientation of membrane proteins found in IIMV is not preserved, resulting in similar rate constants for inward and outward movement. Nevertheless, a faster outward movement of analogues was found in proteoliposomes, suggesting a preferred orientation of a putative flippase in the reconstituted vesicles. The transbilayer distribution of analogues in proteoliposomes is consistent with this. However, we cannot preclude that the amount of analogues in the outer leaflet of proteoliposomes is overestimated since about 10% of the analogues could not be extracted to BSA (see Results). Presumably, these nonextractable analogues are on the inner leaflet of liposomes lacking a rapid flip-flop activity.

Further support for the protein dependence of the fast transbilayer movement of analogues is given by the loss of transport activity upon treatment of proteoliposomes with proteinase K. However, we were not able to eliminate transport-active proteoliposomes completely. Similar observation of a residual pool of transport-active vesicles upon treatment with proteinase K has been made for the flippase activity in the endoplasmic reticulum of rat liver cells [(*10*) and references cited therein]. It has been suggested that this could be due to a flippase which has only on one site of the membrane a proteinase K sensitive segment. Thus, the activities of only those flippases oriented with the sensitive segment to the exterior of proteoliposomes are inactivated by protease. Alternatively, two different flippases with differential protease sensitivity might be present. For the moment, we can only conclude from the protease sensitivity of transport-active vesicles the protein dependence of the fast transbilayer movement of phospholipid analogues in IIMV-derived proteoliposomes. Further studies are warranted, in particular on a more quantitative level, to elucidate the limited protease sensitivity and the remaining pool of vesicles with transport activity.

E. coli is a rapidly growing organism, dividing once every 0.5 h. It requires rapid synthesis of new membrane, and consequently a rapid transbilayer movement of newly synthesized phospholipids. For a bilayer membrane, expansion of one monolayer with respect to the other causes curvatures and extrusion, and eventually vesiculation, a process which can be easily rationalized in the frame of the bilayer couple model (*11*). Therefore, to preserve the stability of inner membranes of *E. coli*, rapid redistribution of phospholipids is of importance. In vesicles composed only of lipids from the inner membrane of *E. coli*, transbilayer movement of phospholipids was slow, as known to be typical for pure lipid membranes (*12*). Thus, proteins acting as a flippase have been assumed to mediate efficient phospholipid flip-flop. According to available data, flippase activities are typical for phospholipids synthesizing membranes of bacteria (*4*−*6*) and eukaryotic cells (*7*, *10*, *13*−*15*). Indeed, in the endoplasmic reticulum of eukaryotic cells such as rat liver cells (*7*) as well as yeast cells (Marx, U., and Herrmann, A., unpublished observation), a rapid protein-dependent transbilayer movement has been unequivocally demonstrated. As we have shown previously by the stopped-flow approach, the half-times of the flip-flop of M-C6-NBD-PC and M-C6-NBD-PE in these cells are of the same order as found here for IIMV and proteoliposomes (*7*).

While the molecular nature of flippases remains to be determined, it is evident that these proteins must possess specific domains that are not typical for membrane proteins in general. Such domains must somehow enable a rapid redistribution of phospholipids between the two leaflets of a membrane bilayer. Transmembrane domains per se are unlikely to be sufficient to mediate a fast flip-flop. Otherwise, a fast transbilayer movement of phospholipids would be a typical feature of all biological membranes. This is not the case. For example, as known for the plasma membrane of metabolic energy-depleted mammalian cells, transbilayer movement of phospholipids is very slow (*16*, *17*). To shed light onto the structure and function of specific protein domains mediating a rapid phospholipid transbilayer movement, the study of synthetic membrane-spanning peptides provides a promising tool (*12*).

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