Dithionite Quenching Rate Measurement of the Inside−Outside Membrane Bilayer Distribution of 7-Nitrobenz-2-oxa-1,3-diazol-4-yl-Labeled Phospholipids†

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ABSTRACT: Measurement of the extent of dithionite quenching of the fluorescence of 7-nitrobenz-2-oxa-1,3-diazol-4-yl- (NBD-) labeled lipids inserted into cellular and organellar membranes has been used to quantify their topological distribution and translocation. This assay provides a straightforward method for determining the fraction of NBD-lipid exposed to the outer leaflet of membranes that are impermeant to dithionite. However, it appears that many, if not all, cellular membranes are relatively permeable to dithionite. The present work describes a method in which the initial rate of dithionite quenching, rather than the extent of quenching, was used to determine the fraction of different NBD-labeled phospholipids exposed to the outer leaflet. This method permits the estimation of the translocation process even in experimental conditions where the membrane is semipermeable to dithionite. This technique was used to measure the translocation of several NBD-labeled phospholipids across two biological membranes: brush border membranes vesicles (BBMV) from rabbit intestine and secretory vesicles (SV) from *sec 6-4* mutant yeast cells. BBMV were shown to passively equilibrate *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)monopalmitoylphosphatidylethanolamine (*N*-NBD-PPE) and 1-palmitoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl]phosphatidylcholine (P-C_6-NBD-PC) to ∼50% in the inner leaflet by a protein-mediated process. In addition, P-C_6-NBD-PC was shown to passively equilibrate across SV to ∼20% in the inner leaflet. The addition of Mg^{2+} increased the amount on the inner leaflet to ∼30% by an unknown mechanism, but no evidence for ATP-dependent inward translocation across the SV was found. In the case of BBMV, several different NBD-phospholipids were translocated from the outer to inner leaflet in a matter of minutes and reached an equilibrium distribution of ∼50% inside and outside. This movement was inhibitable by *N*-bromosuccinimide. The inward translocation rate and distribution of headgroup labeled *N*-NBD-lysophosphatidylethanolamine, having one titratable negative charge, was increased in the presence of an inward basic pH gradient. The same NBD-phospholipids were also translocated across SV to roughly 50% in both leaflets with the exception of NBD-phosphatidic acid, which was passively distributed with 80% in the inner leaflet.

More than 25 years ago, the four major phospholipids of the red cell membrane, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin, were found to be asymmetrically distributed between the two leaflets of the bilayer. Side-specific phospholipases (*1, 2*) and impermeant amino-reactive reagents (*3*−*5*) were used to demonstrate that most, 75−80%, of the sphingomyelin and phosphatidylcholine are localized to the outer leaflet (*1*) whereas about 80% of the phosphatidylethanolamine and essentially all of the phosphatidylserine are localized to the inner leaflet (*1, 2, 4*). This asymmetric distribution of phospholipids has been well established over the years for red cell and platelet membranes and is generally considered to be a property of most, if not all, cells.

To gain insight into the mechanisms by which this asymmetric phospholipid distribution is established, several techniques employing short-chained phospholipids or their spin-labeled or fluorescent-labeled analogues were developed to measure the inward and outward transport rates (*6*−*8*). The replacement of one acyl chain with a truncated fatty acid covalently attached to either a nitroxide spin or fluorescent label increases the water solubility and allows for their rapid introduction into the outer leaflet of the membrane (*9*). Translocation to the inner leaflet is monitored by quenching the NBD[1] fluorescence of those molecules that remain in the outer leaflet with an impermeant reagent (*6, 10*) or rapidly removing (back exchanging) that remaining in the outer leaflet by incubation with an acceptor (BSA or liposomes) (*8*).

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[1] Abbreviations: BBMV, brush border membrane vesicles; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; LUV, large unilamellar vesicles; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBS, *N*-bromosuccinimide; *N*-Rh−PE, *N*-rhodamine−dioleoylphosphatidylethanolamine; *N*-NBD−PPE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-monopalmitoylphosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; P-C_6-NBD-PC, 1-palmitoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl]phosphatidylcholine; P-C_6-NBD-PS, 1-palmitoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl]-phosphatidylserine; PMSF, phenylmethanesulfonyl fluoride; SV, secretory vesicles.

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The usefulness of NBD-labeled lipids for measuring transmembrane transfer was enhanced by the discovery that the relatively impermeant dianion dithionite irreversibly quenched NBD fluorescence (*10*). Assaying the extent of dithionite quenching provides a straightforward method for determining the fraction of NBD-lipid exposed to the outer leaflet of membranes that are impermeant to dithionite. However, although dithionite is essentially impermeant to phospholipid vesicles (liposomes) (*10*), it appears that many, if not all, cellular membranes are relatively permeable to dithionite. Dithionite readily permeates red cell membranes via the anion transporter (band 3) (*11*) and the plasma membranes of cultured cells by an undetermined mechanism (*10, 12*). Thus, proper interpretation of experiments in which the extent of dithionite quenching is used to determine the fraction of NBD-lipid exposed to the outer leaflet is dependent on confirmation of the impermeance of dithionite.

In the present paper, we present a method in which the initial rate of dithionite quenching of NBD-lipids, rather than the extent of quenching, was used to determine the fraction exposed to the outer leaflet. This method does not require complete membrane impermeance of dithionite and therefore should prove useful for the measurement of the bilayer distribution of NBD-lipids in a wide range of cellular membranes that are relatively permeable to dithionite.

EXPERIMENTAL PROCEDURES

Materials and Routine Procedures. POPC, P-C_6-NBD-PC, P-C_6-NBD-PS, and *N*-Rh−PE were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). *N*-NBD-PPE was synthesized by reaction of NBD-chloride with lysopalmitoylphosphatidylethanolamine and purified to >99% as described by Struck et al. (*13*). All lipids were stored at −20 °C, periodically monitored for purity by thin-layer chromatography, and purified when necessary. Phospholipid concentration was determined with a lipid phosphorus assay (*14*). Protein concentration was determined by the Bradford protein assay (*15*).

Preparation of Brush Border Membrane Vesicles. BBMV were prepared from New Zealand White male rabbits using a modification (*16*) of the method of Stevens et al. (*17*). BBMV were resuspended in saline buffer (150 mM NaCl, 20 mM HEPES, and 20 mM MES, pH 7.4) and stored in liquid nitrogen until use. The purity of BBMVs was evidenced by a 15-fold increase in sucrase (*18*) and alkaline phosphatase (*19*) specific activities relative to the crude homogenate.

Preparation of Secretory Vesicles from Yeast. Secretory vesicles were prepared from the *Saccharomyces cerevisiae sec6-4* mutant strain NY17 (MAT**a** *sec6-4 ura3*-52) as described previously by Nakamoto et al. (*20*) with some modifications. The final pellet containing the purified secretory vesicles was resuspended in 150 mM KCl, 5 mM EGTA, and 10 mM Hepes, pH 7.4, centrifuged at 20000*g*, and resuspended in 1−2 mL of the same buffer. Protease inhibitors were added to the lysis buffer and in all subsequent steps at the following concentrations: PMSF, 1 mM; and chymostatin, leupeptin, pepstatin, and aprotinin, 1 μg/mL.

Phospholipid Vesicle Preparation. Large unilamellar vesicles (LUV) were prepared from a mixture of the appropriate phospholipids in chloroform. The phospholipid mixture was dried under a stream of nitrogen and vacuum-desiccated overnight to remove residual solvent. The phospholipid film was resuspended in saline buffer and extruded eight times through two 0.1 μm polycarbonate filters according to the method of Hope et al. (*21*). Suspensions of NBD-phospholipids were prepared by hydrating dried films prepared from chloroform solutions dried under a stream of nitrogen and vacuum-desiccated overnight. The films were hydrated in saline buffer to a concentration of 50 μM by vortex mixing.

Proton Pumping Assay. The integrity of the SV preparations was determined by their ability to create and maintain an ATP- and Mg^{2+}-dependent proton gradient assayed by acridine orange quenching (*20, 22*). For that purpose, SV (200 μg of protein) were suspended in 1 mL of reaction buffer (150 mM KCl, 10 mM HEPES, 2 μM orange acridine, and 5 mM ATP) and placed in a spectrofluorometer cuvette at room temperature. Fluorescence was recorded (excitation 430 nm; emission 530 nm) and the rate and extent of quenching was observed following the addition of 10 μL of 1 M $MgCl_2$. Rapid quenching of 80% of the initial acridine orange fluorescence was indicative of an active and intact SV preparation.

Fluorescence Measurements and Data Analysis of NBD-Labeled Lipids. Fluorescence measurements were performed on an SLM 8000C spectrofluorometer (excitation 475 nm; emission 530 nm). Solutions in the cuvette were stirred continuously with a magnetic stirrer, and the temperature was maintained by a circulating water bath. Digital fluorescence output from the fluorometer was recorded at 1 Hz. The initial rate of the dithionite quenching reaction with NBD was calculated by fitting the initial decay of NBD fluorescence to a single-exponential equation of the general form

$$F(t) = P_1 \exp(-P_2 t) + P_3$$

using a nonlinear least-squares regression analysis (*23*). Only that part of the initial fluorescence decay trace that could be accurately fit by a single-exponential function was selected, and the initial rate was calculated by multiplying P_1 times P_2. This procedure was used to accurately measure the initial rate of fluorescence quenching by dithionite regardless of the complexity of the quenching curve.

RESULTS AND DISCUSSION

On the basis of the experiments presented in Ruetz and Gros (*22*), we attempted to measure the distribution of the fluorescent-labeled phosphatidylcholine, P-C_6-NBD-PC, across BBMV by quenching P-C_6-NBD-PC residing in the outer leaflet with dithionite. Following the addition of micellar P-C_6-NBD-PC to a solution of BBMV, P-C_6-NBD-PC partitioning into the BBMV resulted in an increase in fluorescence (Figure 1). Due to the large dependence of NBD fluorescence on the polarity of its immediate environment (*24*), the fluorescence yield of a water-soluble monomer is ~1% of that for a molecule partitioned into a phospholipid bilayer (*25*). In addition, P-C_6-NBD-PC molecules remaining in micellar aggregates self-quench, resulting in a fluorescence yield less than 1% of the unquenched fluorescence in a phospholipid bilayer (*26*). Thus, partitioning of P-C_6-NBD-PC into the BBMV was accountable for essentially all of the fluorescence measured following the

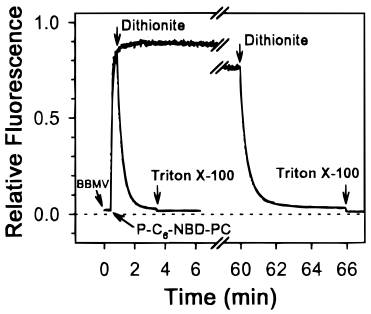

FIGURE 1: Dithionite quenching of P-C_6-NBD-PC in BBMV at 25 °C. BBMV were suspended in 1 mL of HBS in a stirred spectrofluorometer cuvette at 25 °C (final phospholipid concentration 50 μM). An aqueous solution of P-C_6-NBD-PC (10 μL, 50 μM) was added to the cuvette and the fluorescence was continuously recorded. Immediately following stabilization of the fluorescence trace, or after 1 h, 25 μL of 1 M dithionite (pH 10) was added. Following completion of the fluorescence quench, 10 μL of Triton X-100 (10%) was added to the cuvette.

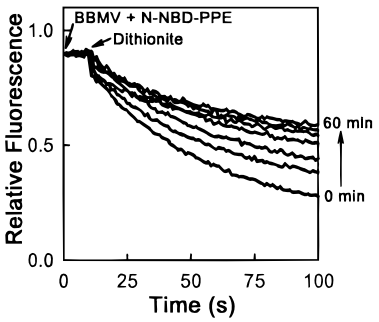

FIGURE 2: Rates of dithionite quenching of *N*-NBD-PPE in BBMV at 2 °C. BBMV were suspended in 7 mL of HBS at 25 °C (final phospholipid concentration 50 μM). An aqueous solution of *N*-NBD-PPE (80 μL, (50 μM) was added and the sample was vortex-mixed for 20 s. Immediately following mixing, a 1 mL aliquot of the suspension was withdrawn, chilled in a water bath (2 °C), and placed in the spectrofluorometer cuvette precooled to 2 °C, and fluorescence was recorded. Following stabilization of the fluorescence trace (~20 s), 25 μL of 1 M dithionite (pH 10) was added, and the decrease of fluorescence was recorded for 100 s. Aliquots of 1 mL of the BBMV suspension were taken at various times and processed in the same way.

addition of P-C_6-NBD-PC to the BBMV, and the decrease in fluorescence following the addition of dithionite was the result of the reduction of P-C_6-NBD-PC residing in one or both of the bilayer leaflets of the BBMV.

After 1 min as well as after 1 h at 25 °C, essentially all of the P-C_6-NBD-PC added to the BBMV was rapidly quenched following the addition of 25 mM sodium dithionite. Assuming that the BBMV were impermeant to dithionite, this result indicated that little or no P-C_6-NBD-PC moved from the outer to inner leaflet of the BBMV during the 1 h incubation at 25 °C. However, this interpretation was inconsistent with previous experiments from our laboratory in which kinetic modeling of the rate and extent of P-C_6-NBD-PC transfer between BBMV and large unilamellar vesicles was used to measure a half-time of 68 s for P-C_6-NBD-PC transmembrane transfer across BBMV at 37 °C (*16*). Given that dithionite has previously been shown to be relatively permeable to red cell (*11*) and cultured cell (*10, 12*) plasma membranes, the assumption that BBMV are impermeant to dithionite may not be correct. If the BBMV were permeable to dithionite, transmembrane movement of NBD-labeled lipids across the BBMV would not be detected by this technique since the dithionite would rapidly react with NBD located in both leaflets of the membrane.

To determine whether the leakage rate of dithionite across the BBMV membrane at 25 °C was too fast to distinguish between the inner versus outer membrane location of NBD-labeled lipids, the temperature of the dithionite quenching reaction was lowered to 2 °C in an effort to slow the rate of dithionite leakage sufficiently to allow the inner and outer membrane pools of NBD-lipid to be distinguished. An example of low-temperature dithionite quenching of the headgroup-labeled lysophosphatidylethanolamine (*N*-NBD-PPE) is shown in Figure 2. *N*-NBD-PPE was added to BBMV at 25 °C and allowed to equilibrate until the fluorescence stabilized (about 30 s; see Figure 1). At this time, an aliquot was removed, rapidly cooled to 2 °C, and placed into the fluorometer sample compartment maintained at 2 °C. Fluorescence was recorded and immediately upon stabilization of the trace, dithionite was added to the cuvette to initiate quenching. The dithionite quenching traces at 2 °C (Figure 2) indicated that both the rate and extent of quenching decreased as a function of incubation time at 25 °C. Thus, there was a reduction in the amount of *N*-NBD-PPE available for dithionite quenching at 2 °C as a function of incubation time at 25 °C. This reduction presumably reflected *N*-NBD-PPE transfer to the inner leaflet of the BBMV.

Conceivably, either the rate or the extent of quenching could be used to quantify the amount of NBD-labeled lipid exposed to the outer surface. Although quantifying the fraction of *N*-NBD-PPE that is quenched by dithionite using impermeant conditions is perhaps the most intuitive approach to quantify the inner versus outer membrane distribution of *N*-NBD-PPE, the slow rate of dithionite leakage even at low temperature complicates this determination. If the rate of dithionite quenching of a single pool of NBD-lipid was described as a single first-order decay reaction, then the inner and outer leaflet pools could be determined by deconvolution of the rate of fluorescence decay into two exponential terms. However, the rate of quenching for NBD-lipids residing exclusively in the outer leaflet of LUVs is complex and is not described as a first-order decay (*10*). Thus, the inner and outer leaflet pools of *N*-NBD-PPE cannot be easily identified by determining the pool sizes corresponding to two different rates of quenching.

An alternative approach for quantifying the fraction of NBD-labeled lipid in the outer leaflet of membranes is to measure the initial rate of dithionite quenching. A plot of the initial rate of dithionite quenching as a function of NBD-lipid concentration in BBMV demonstrated that the initial quenching rate of *N*-NBD-PPE (Figure 3A) and P-C_6-NBD-PC (Figure 3B) was proportional to the amount of NBD-labeled lipid in the membrane. Thus, for membranes that are relatively impermeant to dithionite, the initial rate of dithionite quenching would be expected to be proportional to the amount of NBD-labeled lipid in the outer leaflet. Thus, by measuring the initial rate of dithionite quenching as a function of time, the rate of transfer of NBD-labeled lipid from the inner to outer leaflet could be measured. By assuming that the initial rate measured for the dithionite quenching at the first (zero) time point reflected the rate when all (100%) of the *N*-NBD-PPE or P-C_6-NBD-PC is in the

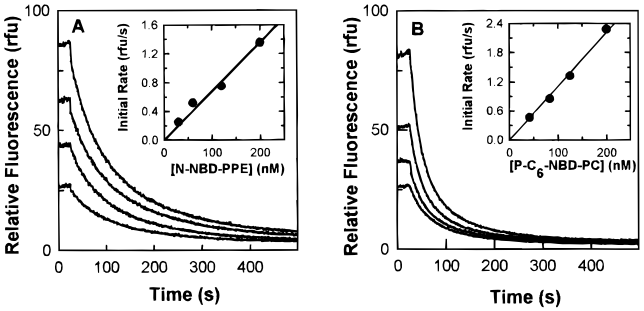

FIGURE 3: Calibration curve for dithionite quenching rate of *N*-NBD-PPE and P-C_6-NBD-PC in BBMV. Different amounts of 50 μM *N*-NBD-PPE (A) or P-C_6-NBD-PC (B) in HBS were added to each of four 1 mL samples of BBMV in HBS (final phospholipid concentration 50 μM). Following addition, the samples were immediately chilled to 2 °C and placed in a precooled spectrofluorometer cuvette, and the fluorescence was recorded. Following stabilization of the fluorescence trace, 25 μL of 1 M dithionite (pH 10) was added. The initial rates of quenching were calculated as described in Experimental Procedures and plotted against the final concentration of NBD-lipid (insets).

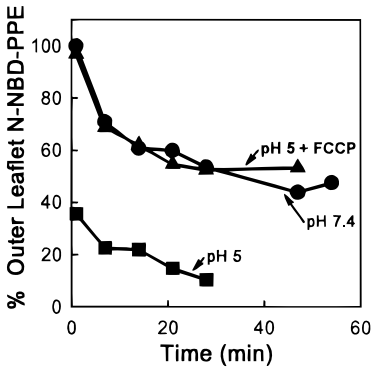

FIGURE 4: Effect of pH gradient on the movement of *N*-NBD-PPE across BBMV. BBMV (final phospholipid concentration 50 μM) were added to tubes containing either HBS at pH 7 (●), HBS at pH 5 (■), or HBS at pH 5 + 1 μM FCCP (▲). *N*-NBD-PPE (final concentration 0.5 μM) was added to the BBMV suspensions, and 1 mL aliquots of the BBMV suspensions were taken immediately and after every 5 min. The rate of dithionite quenching at 4 °C was measured as described in the caption to Figure 2. The initial rate of dithionite quenching measured immediately after the addition of *N*-NBD-PPE to the BBMV suspension at pH 7 was assumed to represent the initial condition where all of the *N*-NBD-PPE was in the outer leaflet. Quenching rates measured for other time points or conditions were plotted in reference to this rate assuming a linear relationship between the rate and concentration in the outer leaflet (set inset in Figure 3).

outer membrane leaflet, the percent remaining at subsequent time points could be calculated from the percent of the maximum initial rate of dithionite quenching. The plot of (●) labeled pH 7.4 in Figure 4 was obtained from the initial rates of the traces presented in Figure 2. This plot demonstrated that the amount of *N*-NBD-PPE available on the outer surface of the BBMV decreased with its time of incubation with the BBMV at 25 °C. The loss of outer leaflet *N*-NBD-PPE presumably reflected its movement to the inner leaflet since no degradation was detected following extraction and TLC analysis of the BBMV suspension.

To test whether the loss of *N*-NBD-PPE available for dithionite was indeed a result of transport to the inner leaflet of the BBMV, the effect of a pH gradient across the BBMV was determined. Headgroup-labeled *N*-NBD-PPE has a single titratable negative charge on the phosphate group (*27*).

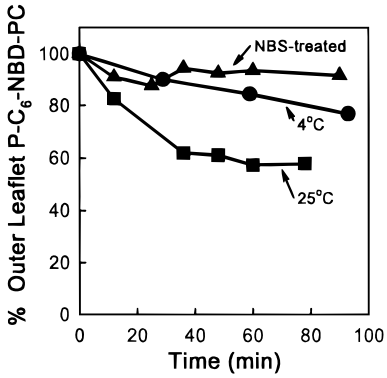

FIGURE 5: Effect of temperature and NBS on P-C_6-NBD-PC movement across BBMV. P-C_6-NBD-PC (0.5 μM) was added to a suspension of BBMV (final phospholipid concentration 50 μM) in HBS at 25 °C (■) or on ice (●). Aliquots of the suspensions (1 mL) were withdrawn immediately following *N*-NBD-PPE addition, and then periodically chilled and placed in a spectrofluorometer cuvette at 2 °C for measurement of the rate of dithionite quenching (see caption to Figure 2). The effect of NBS (▲) was determined by pretreating the BBMV with 1 mM NBS in the dark for 30 min at room temperature, prior to dilution in HBS, pH 7, and addition of *N*-NBD-PPE for the measurement of the dithionite quenching rate.

Thus, one would predict that the neutral species of the *N*-NBD-PPE molecule would readily permeate the BBMV and be trapped inside BBMV, maintaining a basic internal pH relative to the external buffer, as has been shown for phosphatidic acid (*28*). Figure 4 demonstrated that the establishment of a pH gradient across the BBMV (pH 5 outside; pH 7.4 inside) prior to the addition of *N*-NBD-PPE immediately reduced the fraction in the outer leaflet that was available for dithionite quenching as reflected by a decrease in the initial rate. Collapse of the transmembrane pH gradient by the addition of the proton ionophore FCCP returned the time-dependent decrease in the fraction of *N*-NBD-PPE in the outer leaflet to the control rate.

The combination of the aforementioned results—(1) that the initial rate of dithionite quenching of *N*-NBD-PPE was proportional to the amount in the membrane; (2) that the initial rate of dithionite quenching decreased over time to approximately 50% of the maximum initial rate measured initially; and (3) that the predicted inward movement of *N*-NBD-PPE in response to a inside basic pH gradient correlated with a reduction in the initial rate of dithionite quenching—provides strong support for the conclusion that the initial rate of dithionite quenching is proportional to the amount of *N*-NBD-PPE in the outer leaflet of BBMV and that it can be used to monitor the transmembrane flux of NBD-labeled lipids. Based on this interpretation, the experiments presented above support the conclusion that *N*-NBD-PPE is passively equilibrated to equal proportions between the inner and outer leaflets of the BBMV and the proportion of *N*-NBD-PPE on the inner leaflet can be increased in response to a basic inside pH gradient.

The initial rate of dithionite quenching was also used to measure the movement of P-C_6-NBD-PC across the BBMV membrane. The experiment presented in Figure 5 illustrated that P-C_6-NBD-PC passively equilibrated across the BBMV to ~40% in the inner leaflet at 25 °C. This passive equilibration was inhibited at 4 °C as well as by pretreatment of the BBMV with the tryptophan reagent *N*-bromosuccin-

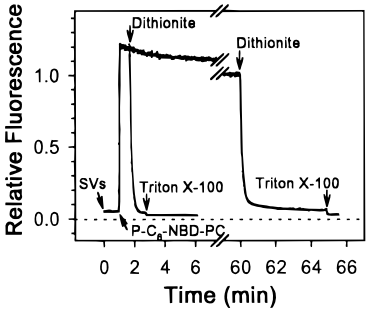

FIGURE 6: Dithionite quenching of P-C_6-NBD-PC in yeast secretory vesicles. SV (final phospholipid concentration 50 μM) were added to 1 mL of HBS in a stirred spectrofluorometer cuvette maintained at 25 °C. Fluorescence was recorded as 10 μL of an aqueous solution of P-C_6-NBD-PC (50 μM) was added to the cuvette. Immediately following stabilization of the fluorescence trace or after 1 h, 25 μL of 1 M dithionite (pH 10) was added to the suspension. Following stabilization of the fluorescence trace, 10 μL of Triton X-100 (10% w/w in HBS) was added to the suspension.

imide (NBS), suggesting that the passive equilibration is protein-mediated.

This technique is limited to membranes that are relatively impermeant to dithionite—that is, conditions must be established in which the rate of quenching is significantly faster than the rate of dithionite flux across the membrane. Furthermore, the choice of NBD-labeled lipid is limited to those for which the rate of membrane association is significantly faster than the rate of transmembrane transfer. This condition can be tested as shown in Figure 1. Partitioning of P-C_6-NBD-PC into the BBMV has reached equilibrium and the resultant fluorescence has stabilized in about 20 s, whereas the rate of P-C_6-NBD-PC translocation to the inner leaflet has a half-time of several minutes.

On the basis of the above results, we concluded that the rate of dithionite leakage across the BBMV at 25 °C was too fast to distinguish inner versus outer leaflet P-C_6-NBD-PC or *N*-NBD-PPE, and therefore the experiment described in Figure 1 did not support the conclusion that P-C_6-NBD-PC remained in the outer leaflet following a 1 h incubation at 25 °C. Since dithionite quenching at 25 °C of essentially all of the P-C_6-NBD-PC fluorescence incorporated into SV (*22*, *29*) and rat liver canalicular membranes (*30*) has been interpreted to reflect a lack of passive equilibration, we decided to measure the passive P-C_6-NBD-PC flux in yeast secretory vesicles (SV) using the initial rate of dithionite quenching at low temperature.

First, we reproduced essentially the same experiment published by Ruetz and Gros (*22*) (Figure 6). Yeast SV were prepared from the *S. cerevisiae sec6-4* strain by the same technique described by Ruetz and Gros and yielded similar results in the acridine orange quenching assay, confirming intact membranes with active Mg^{2+}-ATP-dependent proton pumping (data not shown). In the experiment shown in Figure 6, P-C_6-NBD-PC was added to a cuvette containing SV and allowed to equilibrate at 25 °C for ~30 s or for 1 h, before the addition of dithionite. Essentially all of the P-C_6-NBD-PC fluorescence was accessible to dithionite quenching at both time points, indicating either that essentially all of the P-C_6-NBD-PC remained in the outer leaflet or that dithionite leakage allowed quenching of P-C_6-NBD-PC regardless of its membrane leaflet distribution.

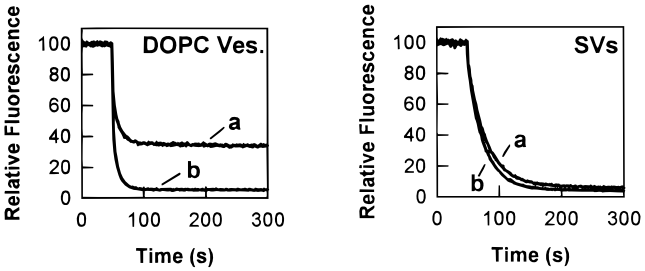

FIGURE 7: Dithionite quenching of P-C_6-NBD-PC in freeze−thawed DOPC vesicles and SV. DOPC extruded vesicles and SV were subjected to three cycles of freeze−thawing in the presence or absence of P-C_6-NBD-PC (final lipid molar ratio 1%). Dithionite quenching at 25 °C was measured as described in Figure 6. Fluorescence traces labeled a were recorded for DOPC vesicles or SV freeze−thawed in the presence of P-C_6-NBD-PC. Fluorescence traces labeled b, were recorded for DOPC vesicles or SV in which an equivalent amount of P-C_6-NBD-PC was added after the freeze−thaw.

To test whether dithionite treatment at 25 °C rapidly quenched P-C_6-NBD-PC residing in the inner leaflet, P-C_6-NBD-PC was introduced into the inner leaflet of SV and DOPC vesicles by freezing and thawing. No significant differences were observed in the rate or extent of dithionite quenching of P-C_6-NBD-PC added prior to (a) or following (b) the freeze−thaw treatment of the SV (Figure 7, SV panel). As a control, DOPC vesicles treated similarly exhibited a clear difference in the rate and extent of P-C_6-NBD-PC fluorescence quenching. The fluorescence of P-C_6-NBD-PC added prior to the freeze−thaw treatment was almost completely quenched by dithionite; whereas for vesicles freeze−thawed in the presence of P-C_6-NBD-PC (a), only 60% of the fluorescence was quenched. These data confirm that dithionite does not penetrate DOPC vesicles and therefore does not quench P-C_6-NBD-PC introduced into the inner leaflet by the freeze−thaw treatment. Assuming that similar freeze−thaw treatment of SV introduced P-C_6-NBD-PC into the inner leaflet, one must conclude that the dithionite rapidly leaked through the SV membrane and quenched P-C_6-NBD-PC residing in the inner leaflet.

Further support for this conclusion was obtained by measuring the passive inward translocation of P-C_6-NBD-PC across SV membranes at 25 °C using the low-temperature, dithionite quenching rate measurement described above. P-C_6-NBD-PC passively equilibrated until at least 20% resided in the inner leaflet (Figure 8). The addition of Mg^{2+} ATP to the SV suspension increased the extent of movement of P-C_6-NBD-PC to the inner leaflet to ~30%. However, an even greater increase was observed by the addition of Mg^{2+} alone in the absence of ATP. The observed increase in the extent of inward P-C_6-NBD-PC movement appears to result from the presence of Mg^{2+} alone. The addition of Ca^{2+} has no effect on the distribution of P-C_6-NBD-PC in this assay. The mechanism for the Mg^{2+} effect is not clear and will require further study. However, these experiments failed to demonstrate ATP-dependent transport of P-C_6-NBD-PC across SV membranes.

In summary, we reported a novel technique for the estimation of the movement of NBD-labeled phospholipids across biological membranes by means of dithionite quenching. The use of low temperature during the quenching process was found to slow the rate of dithionite permeation

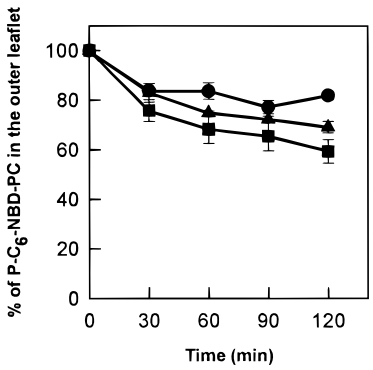

FIGURE 8: Equilibration of P-C_6-NBD-PC across SV at 25 °C. The dithionite quenching assay as described in Figures 2 and 4 was used to measure the percent of P-C_6-NBD-PC in the outer leaflet of SV as a function of time. SV obtained from *sec6-4* mutant cells were suspended in HBS (final phospholipid concentration 50 μM) alone (●) or HBS containing 5 mM $MgCl_2$ (■) or 5 mM $MgCl_2$ and 5 mM ATP (▲) at 25 °C. At the time points shown, P-C_6-NBD-PC was added to the suspension and aliquots of 1 mL were withdrawn, cooled to 2 °C, and placed in a spectrofluorometer. After about 20 s, 20 μL of dithionite (1 M, pH 10) was added and the fluorescence decrease was recorded and analyzed to determine the initial rate of quenching. Error bars represent the standard deviations of the means of four separate experiments.

sufficiently to allow the use of the initial rate of NBD fluorescence quenching to quantify the fraction of NBD-labeled lipid exposed to the outer leaflet. These conditions were found to be necessary because dithionite was found to rapidly permeate BBMV and SV membranes at 25 °C. Thus, the extent of NBD-labeled lipid quenching at 25 °C did not reflect the topological distribution of NBD-labeled lipid in these membranes. Given that other cellular and organellar membranes are likely to have similar degrees of dithionite permeance, the initial rate of dithionite quenching at low temperature as described above should be useful for the further elucidation of the mechanisms for transporting and distributing phospholipids across membranes.

This technique was used in this study to confirm the existence of protein-mediated passive equilibration of *N*-NBD-PPE and P-C_6-NBD-PC across BBMV (*16*). In addition, P-C_6-NBD-PC was shown to passively equilibrate across SV to ~20% on the inner leaflet. The addition of Mg^{2+} increased the amount on the inner leaflet to ~30% by an unknown mechanism, but no evidence for ATP-dependent inward translocation was found.

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