Structural mapping of fluorescently-tagged, functional nhTMEM16 scramblase in a lipid bilayer

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Most members of the TransMEMbrane protein 16 (TMEM16) family are Ca\(^{2+}\)-regulated scramblases that facilitate the bidirectional movement of phospholipids across membranes necessary for diverse physiological processes. The nhTMEM16 scramblase (from the fungus Nectria haematococca) is a homodimer with a large cytoplasmic region and a hydrophilic, membrane-exposed groove in each monomer. The groove provides the transbilayer conduit for lipids, but the mechanism by which Ca\(^{2+}\) regulates it is not clear. Because fusion of large protein tags at either the N or C terminus abolishes nhTMEM16 activity, we hypothesized that its cytoplasmic portion containing both termini may regulate lipid translocation via a Ca\(^{2+}\)-dependent conformational change. To test this hypothesis, here we used fluorescence methods to map key distances within the nhTMEM16 homodimer and between its termini and the membrane. To this end, we developed functional nhTMEM16 variants bearing an acyl carrier protein (ACP) tag at one or both of the termini. These constructs were fluorescently labeled by ACP synthase–mediated insertion of CoA-conjugated fluorophores and reconstituted into vesicles containing fluorescent lipids to obtain the distance of closest approach between the labeled tag and the membrane via FRET. Fluorescence lifetime measurements with phasor analysis were used to determine the distance between the N and C termini of partnering monomers in the nhTMEM16 homodimer. We now report that the measured distances do not vary significantly between Ca\(^{2+}\)-replete and EGTA-treated samples, indicating that whereas the cytoplasmic portion of the protein is important for function, it does not appear to regulate scramblase activity via a detectable conformational change.

Most members of the TMEM16 protein family are Ca\(^{2+}\)-regulated phospholipid scramblases (1–3) that facilitate the bidirectional movement of phospholipids across membranes. TMEM16F, the first member of the family to be recognized as a scramblase, is responsible for the exposure of phosphatidylserine on the surface of activated blood platelets, a key step in blood coagulation (4, 5). Explicit demonstration of the scramblase activity of TMEM16 proteins was achieved when the fungal homologs from Aspergillus fumigatus (afTMEM16) (6) and Nectria haematococca (nhTMEM16) (7) were purified and reconstituted into large unilamellar vesicles and shown to be able to scramble a variety of fluorescently-tagged phospholipids at rates in excess of 10,000 lipids/s in the presence of Ca\(^{2+}\). Scrambling rates were ~100-fold lower in the absence of Ca\(^{2+}\). Both proteins could also transport Cl\(^{-}\) ions in a Ca\(^{2+}\)-dependent manner (6, 8).

The crystal structure of Ca\(^{2+}\)-bound nhTMEM16 revealed it to be a homodimer (Fig. 1A) (7) with a large cytoplasmic region and a hydrophilic, membrane-exposed groove in each monomer located distal to the dimer interface. The groove appears to provide the transbilayer pathway for lipids that was anticipated over a decade ago by the credit card model of lipid scrambling (9). In this model, the headgroups of transiting phospholipids populate the groove, whereas their acyl chains remain associated with the hydrophobic core of the membrane (2, 9, 10). Recent studies identified residues in the groove that are important for scrambling (11–13).

Despite these advances in understanding how TMEM16 scramblases facilitate phospholipid movement, the mechanism by which Ca\(^{2+}\) regulates their scrambling activity is less clear. It seems likely that Ca\(^{2+}\) binding produces a change in protein conformation and/or alters the electrostatic properties of the groove to enable scrambling (14). Interestingly, previous work pointed to the functional importance of the cytoplasmic portion of the protein. Thus, both the scramblase and ion channel activities of afTMEM16 and nhTMEM16 are eliminated when the cytoplasmic portion of the proteins is modified by attachment of a green fluorescent protein (GFP) tag to either the N or C terminus (8). We therefore considered the possibility that the cytoplasmic portion of the protein may play a role in regulating scramblase activity by blocking the lipid pathway in the inactive state and swinging out of the way in the Ca\(^{2+}\)-bound active state to enable lipid passage (Fig. 1B).

To test this model, we reconstituted nhTMEM16 into large unilamellar vesicles and used Förster resonance energy transfer (FRET) to measure the distance of closest approach between a donor fluorophore situated at the N or C terminus of the protein and fluorescent lipid acceptors in the plane of the membrane. We also measured the distance between the N terminus of one monomer and the C terminus of its partner within the nhTMEM16 homodimer. As GFP tagging eliminates scram-
bling activity (8), and site-specific cysteine labeling is currently impractical, we developed a strategy for fluorescently labeling the protein while retaining functionality. A similar labeling strategy was used previously to study ligand-induced conformational changes of the epidermal growth factor receptor relative to the membrane (15). Thus, we generated fusion proteins in which small (~8 kDa) acyl carrier protein (ACP)2 tags and mutant ACP (MCP) tags (16) were appended to the N and/or C terminus of nhTMEM16 (Fig. 1C). We found that the resulting fusion proteins retained their scramblase and channel functions and could be enzymatically labeled with high efficiency using fluorescent coenzyme A (CoA) derivatives. Using these novel fluorescent proteins, we now report that nhTMEM16 reconstitutes into vesicles asymmetrically, such that most molecules are oriented with their cytoplasmic portion in the extravesicular space. Steady-state and lifetime FRET-based structural mapping experiments reveal that the N and C termini of the protein appear to be locked relative to each other, and also with respect to the membrane, during Ca2+-mediated activation.

Results and discussion

ACP-tagged nhTMEM16

The gating model shown in Fig. 1B posits a Ca2+-induced conformational change in the cytoplasmic portion of the protein. To test this model, we set out to use FRET to determine whether the distance between the membrane surface and the cytoplasmically disposed N or C terminus of the protein is affected by Ca2+. Because GFP tagging inactivates both aTMEM16 and nhTMEM16, whereas attachment of a small 48-amino acid sequence (tandem tag consisting of Myc (10 amino acids) followed by a streptavidin-binding peptide (38 amino acids)) to the C terminus of nhTMEM16 does not (8), we considered using the ACP tag for fluorescence labeling (16). ACP is a 77-amino acid tag that can be readily labeled by ACP synthase-mediated transfer of a fluorophore from fluorescent CoA derivatives. We engineered the constructs shown in Fig. 1C, which after expression in Saccharomyces cerevisiae, purification by immobilized metal affinity chromatography, cleavage of the N-terminal His10-EGFP module, and gel filtration yielded well-behaved nhTMEM16 proteins with an N- or C-terminal ACP tag in similar yield as the nontagged protein (Fig. S1). We also generated a doubly-tagged protein (MCP–nhTMEM16–ACP) that will be discussed later.

ACP-tagged nhTMEM16 proteins are functional

We tested the purified ACP-tagged proteins for scramblase and ion channel activity. Scramblase activity was measured using a well-established fluorescence-based assay (Fig. 2A) (6, 17–19). Briefly, large unilamellar vesicles are reconstituted with TMEM16 and fluorescent nitrobenzoxadiazole-conjugated phosphatidylethanolamine (NBD)-PE. NBD-PE is irreversibly converted to nonfluorescent aminobenzoxadiazole-PE by dithionite, a dianionic reductant (6, 18, 20). Because dithionite does not enter the lumen of protein-free liposomes or TMEM16–protein liposomes on the time scale of our experiments (6), it modifies only those NBD-PE molecules that are present in the outer leaflet. Thus, in protein-free liposomes, ~50% of the fluorescence is predicted to be lost on adding dithionite, corresponding to bleaching of the outer-leaflet pool of NBD-PE. In the presence of a functional TMEM16 scramblase, dithionite is predicted to eliminate all fluorescence as NBD-PE molecules from the inner leaflet are scrambled to the outer leaflet. As shown previously (6–8), functional TMEM16 scramblases translocate NBD-PE rapidly in the presence of Ca2+ so that the chemical reduction reaction

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2 The abbreviations used are: ACP, acyl carrier protein; DDM, n-dodecyl-β-D-maltopentanoylsulfate; PC, phosphatidylcholine; FLIM, Fluorescence Lifetime Imaging; MCP, mutant ACP; R18, octadecylrhodamine B; POPE, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; POPG, 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho[1-14C]ethanolamine; Rh-DHPE, rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine; NBD-PE, nitrobenzoxadiazole-conjugated phosphatidylethanolamine; EGFP, enhanced GFP.

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becomes rate-limiting. However, in the absence of Ca\textsuperscript{2+}, scrambling is much slower, and the time course of fluorescence reduction is best described by a double-exponential function with the slow phase corresponding to the rate of scrambling (8).

All three tagged proteins (Fig. 1C) scrambled NBD-PE in a Ca\textsuperscript{2+}-dependent fashion, similar to the activity of the nontagged protein (Fig. 2, B and C, and Table 1). Fluorescence reduction in the presence of Ca\textsuperscript{2+} was well-described in all cases by a single exponential function as reported previously, indicating that the rate of scrambling cannot be distinguished from that of the chemical reduction reaction (Table 1). In contrast, two kinetic components could be distinguished in the absence of Ca\textsuperscript{2+}, corresponding to the expected fast rate of chemical reduction (τ\textsubscript{1} \approx 20 s (Table 1)) and a slower scrambling rate (τ\textsubscript{2} \approx 200–400 s (Table 1)) in the absence of Ca\textsuperscript{2+}. We note that scrambling in the absence of Ca\textsuperscript{2+} appeared to be about 2-fold slower for the C-terminally tagged TMEM–ACP and MCP–TMEM–ACP proteins compared with ACP–TMEM16 and nontagged protein, suggesting mild interference by the C-terminal ACP tag that was not observed for the Myc-SBP tandem tag reported previously (8). The reasons for this are not clear, and as the effect is relatively small, it seems unlikely that C-terminal ACP tagging would affect our structural mapping experiments.

Measurements were made in the absence (black) or presence (red) of Ca\textsuperscript{2+}. The data for each condition were obtained from at least two independent reconstitutions and six traces.

Figure 2. ACP–TMEM16 and TMEM16–ACP have both lipid scramblase and ion channel activity. A, schematic illustration of the phospholipid scramblase assay. See text for details. B, representative traces corresponding to scramblase activity assays for TMEM16, ACP–TMEM16, and TMEM16–ACP. Fluorescence was recorded with a frequency of 1 Hz using λ\textsubscript{ex} = 470 nm and λ\textsubscript{em} = 530 nm. Arrowhead indicates dithionite addition. Traces correspond to protein-free vesicles (blue) and TMEM16 vesicles in the absence (black) or presence (red) of Ca\textsuperscript{2+}. C, scramblase activity of nhTMEM16 and ACP-tagged variants. Activity was calculated as described in Table 1, D, schematic illustration of the ion-channel activity assay. See text for details. E, representative traces corresponding to the ion-channel activity assay. Three sets of traces are shown, each corresponding to vesicles prepared with no protein, ACP–TMEM16, or TMEM16–ACP. Traces correspond to protein-free vesicles (blue) and TMEM16-containing vesicles in the absence (black) or presence (red) of Ca\textsuperscript{2+}. Arrowheads indicate addition of n-octyl-β-D-glucopyranoside to discharge-trapped Cl\textsuperscript{−}. F, trapped Cl\textsuperscript{−} was normalized to the value obtained with protein-free liposome samples that were analyzed in parallel with TMEM16-containing proteoliposomes. Scatter plots show individual measurements along with the mean and S.D. (error bars). Measurements were made in the absence (black) or presence (red) of Ca\textsuperscript{2+}. The data for each condition were obtained from at least two independent reconstitutions and six traces.
Fluorophores themselves are not sensitive to Ca2+, and the fluorescent emission spectra resulting from FRET, we observed that R18 has significant absorbance at the wavelength chosen for donor excitation and that the majority of the observed increase in fluorescence as a function of R18 concentration results from direct excitation of R18 (Fig. S2). Therefore, we used the loss of donor fluorescence as a function of R18 concentration (Fig. 3, C and E) to measure FRET.

<table>
<thead>
<tr>
<th>Construct</th>
<th>% Scramblase activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>τ&lt;sub&gt;f&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>τ&lt;sub&gt;r&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>+EGTA</td>
<td>+Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>+EGTA</td>
</tr>
<tr>
<td>Protein-free</td>
<td>NA</td>
<td>NA</td>
<td>16 ± 3</td>
<td>NA</td>
</tr>
<tr>
<td>nhTMEM16</td>
<td>93 ± 3</td>
<td>90 ± 6</td>
<td>20 ± 3</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>ACP–TMEM16</td>
<td>91 ± 9</td>
<td>46 ± 8</td>
<td>24 ± 6</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>TMEM16–ACP</td>
<td>95 ± 3</td>
<td>32 ± 2</td>
<td>18 ± 3</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>MCP–TMEM16–ACP</td>
<td>94 ± 2</td>
<td>35 ± 6</td>
<td>18 ± 4</td>
<td>14 ± 3</td>
</tr>
</tbody>
</table>

<sup>a</sup> % Scramblase activity = 100 × (F<sub>TMEM16</sub> (t = 100 s) − F<sub>PF</sub> (t = 100 s))/F<sub>TMEM16</sub> + Ca<sub>2+</sub> (t = 900 s) − F<sub>PF</sub> (t = 900 s), where F<sub>PF</sub> (t = 100 s) and F<sub>TMEM16</sub> (t = 100 s) represent the fluorescence of protein-free vesicles and TMEM16-containing vesicles, respectively, 100 s after dithionite addition. F<sub>PF</sub> (t = 900 s) and F<sub>TMEM16</sub> + Ca<sub>2+</sub> (t = 900 s) represent the fluorescence of protein-free vesicles and TMEM16-containing vesicles, in the presence of Ca<sup>2+</sup>, 900 s after dithionite addition. Values of F<sub>TMEM16</sub> + Ca<sub>2+</sub> (t = 900 s) − F<sub>PF</sub> (t = 900 s) ranged from 0.36 to 0.50, similar to previous reports.

<sup>b</sup> Time constants τ<sub>f</sub> and τ<sub>r</sub> were obtained from a double exponential fit of the fluorescence reduction traces obtained on adding dithionite (for example, Fig. 2B). For protein-free liposomes and TMEM16-containing liposomes in the presence of 0.5 mM Ca<sup>2+</sup>, the traces were well-represented by a single exponential decay, hence only τ<sub>f</sub> is reported for these samples.

Distance of closest approach between the ACP tag in nhTMEM16 variants and the plane of the membrane

We fluorescently labeled ACP–TMEM16 and TMEM16–ACP using CoA–488 and reconstituted the labeled proteins into liposomes containing up to 2.0 mol % of R18 lipids to carry out FRET measurements as schematically illustrated in Fig. 3A. The fluorophores themselves are not sensitive to Ca<sup>2+</sup> or EGTA (see “Experimental procedures”). Fluorescence emission spectra (λ<sub>ex</sub> = 480 nm) of samples prepared in the presence of Ca<sup>2+</sup> or EGTA revealed a progressive increase in the magnitude of R18 emission as a function of the amount of reconstituted R18 (Fig. 3, B and D). Although a portion of this signal may be due to sensitized emission resulting from FRET, we observed that R18 has significant absorbance at the wavelength chosen for donor excitation and that the majority of the observed increase in fluorescence as a function of R18 concentration results from direct excitation of R18 (Fig. S2). Therefore, we used the loss of donor fluorescence as a function of R18 concentration (Fig. 3, C and E) to measure FRET.

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We quantified donor fluorescence (value at 520 nm, Fig. 3, C and E) in the presence of acceptor ($F_{DA}$) and also after dispersal of the acceptor by treating the vesicles with Triton X-100 ($F_{p}$, donor fluorescence in the absence of acceptor). These data were corrected using an identically prepared liposome sample lacking fluorophores and used to compute the ratio of the quantum yield (Q) of the donor in the absence versus presence of the acceptor ($Q_D/Q_{DA}$). In parallel, the surface density of R18 molecules in the membrane was calculated by measuring bulk phospholipid with a colorimetric assay after hydrolysis to P1 and quantifying R18 spectroscopically. Representative plots of $Q_D/Q_{DA}$ versus acceptor density are shown in Fig. 4. The slope of the plots is proportional to the inverse fourth power of the distance of closest approach ($L$) between the donor fluorophore on the protein and the plane of R18 acceptors in the membrane (see “Experimental procedures”) (22, 23). $L$ values of ~50 Å were obtained with both ACP–TMEM16 and TMEM16–ACP, in the presence or absence of Ca$^{2+}$ (Fig. 4, Table 2), consistent with the estimated position of the cytoplasmic portion of the protein relative to the plane of the membrane (Fig. 1) (7). We note that this estimate includes approximations for small sections of the sequence at the N and C terminus that could not be resolved in the nhTMEM16 crystal structure (7) and for the fluorophore-modified ACP tag.

The plots shown in Fig. 4 suggest Ca$^{2+}$-dependent changes in the position of the ACP tag relative to the membrane, with the N terminus moving further away from the bilayer in the presence of Ca$^{2+}$, whereas the C terminus moves toward the bilayer. To verify these results, we repeated these experiments and also used a different membrane lipid Rh-DHPE as a fluorescent acceptor. We obtained multiple data sets that are summarized in Table 2. Although on average the N terminus appears to be 5 Å further from the membrane in the presence of Ca$^{2+}$, the difference between the +Ca$^{2+}$ and +EGTA measurements is not significant ($p > 0.1$). Likewise, we did not find a significant change in the position of the C-terminal tag relative to the membrane in the +Ca$^{2+}$ and +EGTA conditions (Table 2).

### FLIM–FRET measurements with doubly-tagged nhTMEM16

We next attempted to measure the distance between the N terminus of one monomer and the C terminus of its partner monomer in the TMEM16 heterodimer (the N and C termini of an individual monomer are too far apart for FRET-based detection) (Fig. 1A) as a function of Ca$^{2+}$. We purified MCP–TMEM16–ACP (Fig. 1C and Fig. S1) and verified that it had scramblase activity (Table 1). We exploited the difference in specificity of ACP synthase and its *Bacillus subtilis* counterpart (SFP synthase) to label sequentially the ACP tag with 488 dye and the MCP tag with 547 dye as described under “Experimental procedures.” FLIM with phasor representation was then used to measure FRET efficiency. For each sample, donor alone (D) and donor with acceptor (D + A), a lifetime image of the solution was acquired as described under “Experimental procedures.” Our FLIM system uses a digital frequency domain acquisition card, with a 491-nm excitation laser at a base frequency of 20 MHz, and six harmonics to describe the frequency response to detect FRET (24, 25). Data were analyzed using ISS FLIM software by the phasor plot at 40 MHz and by the multifrequency lifetime fit analyses at 20–140 MHz.

In a phasor plot, the sine and cosine transform of the time decay is calculated from the phase and modulation value obtained at every pixel of the FLIM image. This then places

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**Table 2**

<table>
<thead>
<tr>
<th>TMEM16 donor</th>
<th>Membrane acceptor</th>
<th>$R_0$ (Å)</th>
<th>$L$ (Å)</th>
<th>Slope ± S.E.$^a$</th>
<th>+Ca$^{2+}$</th>
<th>+EGTA</th>
<th>+Ca$^{2+}$</th>
<th>+EGTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>488-TMEM16</td>
<td>R18</td>
<td>57.5</td>
<td>7770 ± 660</td>
<td>7000 ± 500</td>
<td>52.0 ± 1.0</td>
<td>50.0 ± 1.0</td>
<td>53.0 ± 1.0</td>
<td>46.5 ± 0.5</td>
</tr>
<tr>
<td>488-TMEM16</td>
<td>Rh-DHPE</td>
<td>57</td>
<td>6620 ± 1600</td>
<td>6100 ± 1400</td>
<td>53.0 ± 3.0</td>
<td>51.0 ± 1.0</td>
<td>54.0 ± 3.0</td>
<td>44.0 ± 3.0</td>
</tr>
<tr>
<td>TMEM16-488</td>
<td>R18</td>
<td>57.5</td>
<td>7600 ± 485</td>
<td>7600 ± 485</td>
<td>52.0 ± 1.0</td>
<td>58.5 ± 0.7</td>
<td>48.5 ± 0.5</td>
<td>52.0 ± 0.5</td>
</tr>
</tbody>
</table>

$^a$ The slope was obtained by linear regression of $Q_D/Q_{DA}$ versus acceptor density data (e.g. Fig. 4); S.E. = standard error of the fit.

$^b$ $L$ was calculated from the slope by using Equation 2 (see “Experimental procedures”). Each row of $L$ values indicates an independent experiment involving a unique protein preparation and a series of reconstituted proteoliposomes containing different amounts of acceptor lipid. Data are given as mean ± S.D. from replicate measurements of each reconstituted vesicle sample. Forster radius ($R_0$) for each donor–acceptor pair was determined by using Equation 3 (see “Experimental procedures”) with a value of $\Phi$ (quantum yield of donor-labeled protein) = 0.44. Fluorescence measurements were carried out using $\lambda_{em} = 520$ nm, and $\lambda_{ex} = 480$ and 492 nm, respectively, for R18 and Rh-DHPE.
each pixel in the homogeneous image at a specific location on the universal semi-circle phasor plot, indicating a single exponential decay, or within the semi-circle, indicating a multiexponential decay (24, 25). A graphical representation of the data for CoA-488–labeled MCP–TMEM16–ACP (D) and CoA-488/CoA-547–labeled MCP–TMEM16–ACP (D/H11001A) samples is shown in Fig. 5, A and B. Pixels for the D/H11001A sample appear along the straight-line trajectory between the donor lifetime of tD on the semi-circle and a short lifetime tDA2. The D/H11001A sample pixels are shifted 15% away from the donor lifetime toward the 1-ns lifetime tDA2, indicating that 15% of the intensity is due to a short fluorescence lifetime of tDA2, consistent with FRET, whereas 85% of the light comes from the longer donor-only lifetime of ~3.6 ns (tDA1). The presence or absence of Ca2+ had no effect on the fractional population displaying FRET.

Analysis of the phase and modulation data yielded lifetimes consistent with the phasor results. Data for the CoA-488–labeled MCP–TMEM16–ACP (D) fit well to a single exponential decay with a lifetime of ~3.8 ns (tD) in the presence and absence of Ca2+ (Fig. 5C and D, and Table 3), whereas data for the CoA-488/CoA-547–labeled MCP–TMEM16–ACP (D + A) fit well to a double-exponential decay with a combination of two emitting species at 3.6 ns (tDA1) and 1.2 ns (tDA2) in the presence of Ca2+ (Fig. 5E and Table 3) and 3.6 ns (tDA1) and 1.0 ns (tDA2) in the absence of Ca2+ (Fig. 5F and Table 3). The fractional population of molecules undergoing FRET recovered from the fits of the frequency response curves was found to be near 30% in all cases. Because the lifetime of the second component is shorter due to FRET, this population contributes only 15% of the intensity, consistent with the position of the pixels in the phasor plots. The FRET efficiency deduced from the donor-only lifetime tD and the short lifetime observed in the donor acceptor samples, tDA2, was 73%, calculated using Equation 7 ("Experimental procedures," with tDA2 = 1.0 ns). The distance between the donor and the acceptor for this FRET fraction was estimated to be ~55 Å, calculated using Equation 8 and an R₀ value of 65 Å (see under "Experimental procedures").

Based on the labeling efficiency (~80%; see under "Experimental procedures"), the maximal population of MCP–TMEM16–ACP molecules that can undergo FRET is ~60–65%. However, only 30% of all molecules, i.e. ~50% of the doubly-labeled population, undergoes FRET. Fluorescence polarization measurements on the donor indicated that it was
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Table 3

Fluorescence lifetime measurements of nhTMEM16 by multifrequency phase/modulation

<table>
<thead>
<tr>
<th>Sample</th>
<th>τD ± S.D.</th>
<th>τDA1 ± S.D.</th>
<th>τDA2 ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Ca²⁺</td>
<td>+EGTA</td>
<td>+Ca²⁺</td>
</tr>
<tr>
<td>D⁶</td>
<td>3.81 ± 0.05</td>
<td>NA</td>
<td>3.79 ± 0.06</td>
</tr>
<tr>
<td>DA³</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Lifetimes of donor (τD) and donor with acceptor (τDA1) were analyzed by multifrequency lifetime fitting. Donor-alone (τD) lifetimes were best fit with a single-exponential function, whereas the donor + acceptor samples were fit to a double-exponential decay with two fractions. τD, τDA1, and τDA2 (τDA2 is the lifetime of molecules that do not undergo FRET) and τDA1 is the lifetime of molecules that undergo FRET. S.D. = standard deviation from the fits of n = 20 individual measurements from two independent protein preparations.

† D = MCP-TMEM16-488 (donor-only construct); DA = 547-TMEM16-488 (donor + acceptor construct).

‡ The numbers in parentheses correspond to the fractional population of the lifetime component τDA1 or τDA2.

freely rotating, and hence, the relative orientation of donor and acceptor dipoles should not be a limiting factor for FRET efficiency. However, it is possible that we did not observe FRET for the full potential protein population because the protein undergoes conformational dynamics that are slow on the time scale of fluorescence, leading to a situation in which the donor and acceptor are not within the appropriate distance necessary for FRET for a significant proportion of the molecules in the ensemble. Another possibility is that there is an appreciable acceptor fluorophore population in the dark state, which is consistent with the small sensitized emission signal seen in steady-state fluorescence (data not shown). Either of these possibilities would have lowered the fraction of observed FRET donor fluorophore lifetimes (26).

TMEM16 reconstitutes asymmetrically into proteoliposomes

In the course of our experiments on lipid scrambling by the different TMEM16 constructs, we noticed that the Atto-488 dye is bleached by dithionite. We made use of this observation to determine the orientation of ACP-tagged TMEM16 proteins in proteoliposomes. We reconstituted CoA-488–labeled ACP–TMEM16 or TMEM16–ACP into liposomes and treated the vesicles with dithionite after determining their initial fluorescence. As shown in Fig. 6A, >80% of the fluorescence was lost on dithionite treatment; complete loss of fluorescence was subsequently observed on adding Triton X-100 to disrupt the vesicle membrane. As dithionite does not enter TMEM16-containing proteoliposomes (6), these results indicate that the majority of the TMEM16 dimers are reconstituted asymmetrically such that their N and C termini are accessible to the extravesicular space (Fig. 6B).

Figure 6. Asymmetric reconstitution of TMEM16. A, CoA-488–labeled ACP-tagged nhTMEM16 was reconstituted into liposomes, and its fluorescence was monitored as a function of time in a fluorescence spectrometer (λex = 492 nm and λem = 520 nm). Dithionite was added as indicated, followed by addition of Triton X-100 to disrupt the vesicles. B, loss of ~80% of fluorescence was observed for both CoA-488–labeled ACP–TMEM16 and TMEM16–ACP in A, indicating that at least 80% of the proteins are oriented in the vesicles as shown, with their cytoplasmic portion (N and C termini) facing the extravesicular space.

Conclusion

We tested the hypothesis that a conformational change in the cytoplasmic region of TMEM16 scramblases is involved in controlling access to the transbilayer groove that forms the highly lipid and ion translocation pathway. This hypothesis is rooted in previous observations that the attachment of GFP tags to the N or C terminus of the fungal scramblases afTMEM16 and nhTMEM16 abrogates their scramblase activity without impacting overall protein structure, including the ability to dimerize (8). Our FRET-based analyses reveal that the distance of closest approach between the ACP tag at the N or C terminus of nhTMEM16 and the membrane does not vary significantly between Ca²⁺-replete and EGTA-treated samples. Likewise, the distance between the N terminus of one monomer and the C terminus of its partner does not change detectably in the presence of Ca²⁺, although for most molecules this distance could not be estimated by FRET because the termini are either too far apart or a significant population of the acceptor is in the dark state as discussed above. Our data therefore suggest that Ca²⁺-mediated activation of TMEM16 scramblases does not appear to require major movement of the cytoplasmic portion of the protein. It remains possible that a more nuanced movement of the cytoplasmic part of the protein is sufficient to control the lipid and ion pathway and that we could not detect it within the limitations of our measurements and/or that the relative movements of the fluorophores are such that they do not result in FRET changes. There are also other explanations that would account for our results. For example, it is known that detergents can activate membrane transporters (27). Thus, it is possible that detergent solubilization (the first step in obtaining purified protein after expression in yeast) converts nhTMEM16 into a partially active conformation and that this conformation is preserved even after detergent removal and reconstitution. This is consistent with the ability of nhTMEM16 to scramble lipids at a low rate even in the absence of added Ca²⁺. Although Ca²⁺ addition is necessary for full activation, it may be that the most significant conformational changes have already occurred irreversibly during protein purification accounting for our inability to detect these further Ca²⁺-mediated changes by FRET.
Recent structures of TMEM16A obtained by cryo-EM (28–30) and molecular dynamics simulations of nhTMEM16 (11) shed some light on this issue. TMEM16A, the founding member of the TMEM16 family, functions as a Ca\(^{2+}\)-activated Cl\(^{-}\) channel but lacks scramblase activity (1). The cryo-EM structures of TMEM16A indicate that it is a homodimer, but, unlike nhTMEM16, its component monomers interact minimally and are not intertwined on the cytoplasmic side (Fig. 1A). The structures of Ca\(^{2+}\)-bound and apo-TMEM16A (29, 30), both in detergent and after reconstitution into nanodiscs, suggest that it is activated when a transmembrane helix (TM6) that lines the pore twists toward the Ca\(^{2+}\)-binding site, directly interacting with the bound Ca\(^{2+}\) ions while simultaneously exposing the cytoplasmic entryway to the pore. The structural conservation between the channel-only TMEM16A protein and the channel/scramblase nhTMEM16 protein suggests that the activation mechanism proposed for pore-opening in TMEM16A may also control opening of the groove in nhTMEM16 to enable its scrambling function. Molecular dynamics simulations of nhTMEM16 indicate specific conformational changes in TM4 during Ca\(^{2+}\) activation (11). Because both structural and in silico studies suggest that Ca\(^{2+}\) activation may require conformational changes in TM domains, we engineered variants of nhTMEM16 in which a mini-ACP tag sequence (GDSLSWLLRLLNG) was inserted in the cytoplasmic loop between TM6 and TM7, bracketed by different spacer sequences. Our aim was to tag the apparently mobile end of TM6 with a fluorophore in order detect the changes in conformation that were suggested by the cryo-EM structures of the TMEM16A channel. However, these experiments were not successful as the expressed proteins could only be recovered as aggregates. The resolution of this issue will require the introduction of site-specific reagents, a goal for future work.

Finally, we note that our study introduces new tools for the study of TMEM16 scramblases. Thus, we report the first functional, fluorescently tagged variants of this class of scramblase proteins. We have already used these novel reagents in establishing the asymmetric reconstitution of TMEM16 (Fig. 6), a result that will be important in understanding macroscopic rate constants obtained from scramblase activity data.

**Experimental procedures**

**Materials**

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmityl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (sodium salt) (POPG), egg \(\alpha\)-phosphatidylcholine (60% purity, egg PC), and 1-myristoyl-2-[6–(7-nitro-2,1,3-benzoazadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphoethanolamine (NBD-PE) were obtained from Avanti Polar Lipids. \(n\)-Dodecyl-\(\beta\)-D-maltopyranoside (DDM) was from Anatrace; \(n\)-octyl-\(\beta\)-D-glucopyranoside was from Affymetrix; BioBeads SM2 adsorbent was from Bio-Rad; and ACP synthase, SFP synthase, CoA-488, and CoA-547 (based on Atto-488 (ATTO-TEC) and DY-547 (Dyomics), respectively) were from New England Biolabs. Octadecylrhodamine B chloride (R18) and Rh-DHPE were from ThermoFisher Scientific and Setareh Biotech, respectively. All other chemicals were from Sigma.

**Buffers**

The following buffers were used: Buffer A (50 mM HEPES, pH 7.6, 150 mM NaCl, 0.5 mM CaCl\(_2\), EDTA-free protease inhibitor mixture (Roche Applied Science, 1 tablet per 50 ml of buffer)); Buffer B (10 mM HEPES, pH 7.6, 150 mM NaCl, 5% (w/v) glycerol, 0.025% (w/v) DDM, 5 mM CaCl\(_2\)); Buffer C (5 mM HEPES, pH 7.6, 150 mM NaCl, 0.025% (w/v) DDM, and 3 mM CaCl\(_2\)); Buffer D (20 mM HEPES, pH 7.4, 300 mM KCl); and Buffer E (1 mM KCl, 300 mM sodium glutamate, 20 mM HEPES, pH 7.4).

**nhTMEM16 constructs**

Unique EcoRI and BamHI restriction sites were introduced at the 5′ and 3′ ends of the open reading frame in a construct encoding His\(_{10}\)-EGFP–nhTMEM16. The ACP-tag sequence, generated by EcoRI/BamHI restriction digestion of the pACP-tag(m)-2 vector (New England Biolabs) was ligated to the nhTMEM16 sequence to generate constructs encoding His\(_{10}\)-EGFP–ACP–TMEM16 and His\(_{10}\)-EGFP–TMEM16–ACP. The double-tagged construct (MCP–TMEM16–ACP) was generated in two stages. The ACP tag was converted to MCP by introducing mutations into the pACP-tag(m)-2 plasmid corresponding to D36T and D39G, and after sequence verification, the MCP-encoding fragment was recovered by PCR and inserted at the 5′ end of the TMEM16–ACP gene by using the Gibson assembly cloning kit (New England Biolabs) according to the manufacturer’s protocol.

**Expression and purification of nhTMEM16 constructs**

Protein expression and purification were carried out as described previously, using S. cerevisiae FGY217 (MATa ura3-52 lys2A201 pep4A) cells transformed with a modified pYES2/CT vector containing the gene of interest and a URA3 marker for selection of transformants (7, 8). Briefly, cells were grown to \(A_{600} \sim 0.7–0.8\) at 30 °C, before inducing expression of the TMEM16 construct with 2% (w/v) galactose at 25 °C and harvesting after 40 h. Cells were resuspended in Buffer A (supplemented with DNase I and 1 mM MgCl\(_2\)) and disrupted using an EmulsiFlex-C3 homogenizer at 25,000 p.s.i. After low-speed centrifugation to remove unbroken cells, membranes were pelleted by ultracentrifugation at 40,000 rpm (1.5 h, 4 °C) in a 45 Ti rotor (Beckman). Membranes were homogenized in Buffer A and solubilized in 1% (w/v) DDM at 4 °C for 1.5 h. Insoluble material was removed by centrifugation at 40,000 rpm for 0.5 h with 45 Ti rotor. The supernatant was incubated with 1 ml of nickel-nitrioltriacetic acid resin in the presence of 15 mM imidazole at 4 °C for 1.5 h. The resin was washed with 10 ml of Buffer B containing 50 mM imidazole. The protein was eluted with Buffer B modified to contain 400 mM imidazole.

The His\(_{10}\)-EGFP tag was cleaved off by treating the purified protein with human rhinovirus 3C protease (overnight incubation, 4 °C, protein/protease mass ratio 1:1). After protease cleavage, the TMEM16 construct was purified by size-exclusion chromatography on a Superdex-200 gel-filtration column run in Buffer C.

The purified protein was taken for fluorescence labeling with CoA-488. The reaction was carried out at room temperature for 2 h using a molar ratio of TMEM16/CoA-488/SFP-synthase = 1:2:0.2 with 10 mM MgCl\(_2\). The labeled protein was re-purified...
Structural mapping of nhTMEM16 scramblase

by Superdex-200 gel–filtration chromatography using Buffer C. The degree of labeling was calculated from the absorbance spectrum of the labeled protein as \( (\frac{A_{\text{dye}}}{A_{\text{protein}}}) \times 100\), where \( A_{\text{dye}} = 73,000 \text{ M}^{-1} \text{ cm}^{-1} \) for CoA-488 at 495 nm, \( A_{\text{protein}} = 129,000 \text{ M}^{-1} \text{ cm}^{-1} \) at 280 nm calculated from the protein sequence using the ExPASy-ProtParam tool (https://web.expasy.org/protparam/), and \( A = \) absorbance at 280 and 495 nm, for protein and dye, respectively. Labeling efficiency (mean ± S.D.) was 79 ± 16% \((n = 8)\) and 75 ± 12% \((n = 2)\) for ACP–TMEM16 and TMEM16–ACP, respectively.

Proteoliposomes

Fluorescently labeled nhTMEM16 variants were reconstituted into proteoliposomes as described previously \((6, 8, 31)\). Briefly, lipid stock solutions were mixed to obtain 20 mg of lipids (~27 μmol) in a molar ratio of three parts (3 POPE, 1 POPG) to one part 60% egg PC and evaporated to dryness under a gentle stream of nitrogen. Fluorescent lipids were included in the lipid mixture before evaporating the solvent: \( C_6\)-NBD-PE \((0.5 \text{ mol} %) \) was included in samples being prepared for scramblase assays, and R18 or Rh-DHPE \((0.5–2.0 \text{ mol} %) \) was included for FRET experiments. The obtained lipid film was hydrated with 1 ml of Buffer D, supplemented with 35 mM CHAPS, at room temperature for 30 min. The resulting lipid suspension was sonicated in a bath sonicator until it appeared clear, before adding protein (5 mg of protein per 1 mg of lipid mixture). After 30 min of incubation at room temperature, detergent was removed by stepwise addition of Bio-Beads SM-2 adsorbent \((160 \text{ mg of Bio-Beads per ml, in four exchanges at 4 °C})\). Liposomes were subjected to three freeze–thaw cycles before being extruded 21 times through a 400-nm membrane filter (using a mini-extruder (Avanti Polar Lipids)) in the presence of 0.5 mM Ca(NO₃)₂ or 2 mM EGTA immediately prior to being taken for scramble or flux assays, or for FRET measurements. Dynamic light scattering measurements (Zetasizer Nano-S, Malvern Instruments) indicated that the reconstituted proteoliposomes had an average diameter of ~345 nm.

Scramblase assay

Scramblase activity was measured as described previously \((6, 8)\). Briefly, 20 μl of extruded \( C_6\)-NBD–PE-containing liposomes were diluted into 2 ml of Buffer D (with 50 mM NaCl instead of 20 mM HEPES, pH 7.4) in the presence of 0.5 mM Ca(NO₃)₂ or 2 mM EGTA in a stirred cuvette at room temperature. Fluorescence \((\lambda_{\text{em}} = 470 \text{ nm and } \lambda_{\text{em}} = 530 \text{ nm}) \) was monitored in a fluorescence spectrometer (Photon Technology International, HORIBA Scientific) at a frequency of 1 Hz as a function of time. After obtaining a stable initial fluorescence signal, 40 μl of freshly prepared sodium dithionite in 0.5 M Tris–HCl, pH 10 (20 mM final concentration), was added, and the resulting time-dependent fluorescence loss was monitored for 900 s.

Flux assay

Flux assays to measure channel activity were done as described previously \((6, 8)\). Briefly, 100 μl of extruded liposomes were passed through a Superdex G-50 desalting column equilibrated in Buffer E in the presence of 0.5 mM Ca(NO₃)₂ or 2 mM EGTA. The flow-through was diluted into 2 ml of Buffer E, and Cl⁻ content was measured using an AgCl electrode. The total Cl⁻ content of the liposomes was determined by disrupting the liposomes with 40 μl of 1.5 M n-octyl-β-D-glucopyranoside. The change in Cl⁻ content was normalized to the value obtained for protein-free liposomes in the presence of 0.5 mM NaCl prepared from the same batch of lipids.

Steady-state fluorescence measurements

Measurements were performed on continuously stirred samples at room temperature, using a Photon Technology International fluorescence spectrometer. Freshly extruded R18- or Rh-DHPE–containing proteoliposomes \((5 \mu l) \) were diluted to 2 ml in Buffer D in the presence of 0.5 mM Ca(NO₃)₂ or 2 mM EGTA. Emission spectra were recorded by exciting the donor at \( \lambda_{\text{ex}} = 480 \text{ nm for experiments with R18, and } \lambda_{\text{ex}} = 492 \text{ nm for Rh-DHPE. Spectra were corrected for light scattering by subtracting the signal from unlabeled liposomes lacking donor and acceptor fluorophores.}

After measurement of donor emission \( (F_{\text{DA}}, \text{fluorescence of donor in the presence of acceptor}) \), Triton X-100 \((\text{final concentration } 0.1\% \text{ (w/v)}) \) was added to solubilize the vesicles and disperse the acceptors. The fluorescence of the Triton X-100–treated sample, \( F_D \), was considered to represent the fluorescence of donor in the absence of acceptor. Values of \( F_D \) and \( F_{\text{DA}} \) were corrected by subtracting the signal, \( F_U \), from identically treated unlabeled liposomes.

We determined that the fluorophores were not themselves sensitive to the presence of \( \text{Ca}^{2+} \) or EGTA by measuring the fluorescence of Triton X-100–solubilized vesicles containing either CoA-488–labeled, ACP-tagged TMEM16, or R18 in the presence and absence of \( \text{Ca}^{2+} \) (for the 488 fluorophore, we used \( \lambda_{\text{ex}} = 480 \text{ nm and } \lambda_{\text{em}} = 520 \text{ nm; for R18, we used } \lambda_{\text{ex}} = 560 \text{ nm and } \lambda_{\text{em}} = 585 \text{ nm} \)). The ratio of fluorescence in the presence \( \text{versus} \) absence of \( \text{Ca}^{2+} \) was 0.99 ± 0.15 (mean ± S.D., \( n = 8 \)), four independent reconstructions and 1.00 ± 0.10 (mean ± S.D., \( n = 10 \), two independent reconstructions), for the 488 fluorophore and R18, respectively.

The ratio of the quantum yield of the donor in the absence \( (Q_D) \) or presence \( (Q_{\text{DA}}) \) of acceptor was calculated from the fluorescence data according to Equation 1.

\[
\frac{Q_D}{Q_{\text{DA}}} = \frac{(F_D - F_U)}{(F_{\text{DA}} - F_U)} \tag{Eq. 1}
\]

Distance of closest approach \((L)\)

\( L \) was calculated from \( \frac{Q_D}{Q_{\text{DA}}} \) and the surface density of the acceptor in the membrane. The surface density of acceptor was determined by the following: (i) quantifying bulk lipids by measuring \( P_l \) released after acid hydrolysis \((32)\); (ii) quantifying the acceptor by measuring absorbance at 560 nm using a molar extinction coefficient of 100,000 M⁻¹ cm⁻¹ for R18 and 119,400 M⁻¹ cm⁻¹ for Rh-DHPE; and (iii) assuming that a phospholipid molecule has a cross-sectional area of 70 Å² and that the acceptors are distributed uniformly and randomly on the surface of the bilayer \((33)\). The slope of a plot of \( \frac{Q_D}{Q_{\text{DA}}} \) versus the surface density of acceptors \((\text{Å}^{-2})\) was used to calculate \( L \) according to Equation 2 \((22, 23, 34, 35)\).

\[
\text{slope} = \frac{(\pi R_0^2)}{(2L^3)} \tag{Eq. 2}
\]
The Förster distance \( R_0 \) in Å units for donor and acceptor pairs was calculated from the degree of overlap between the donor emission spectrum and acceptor absorption spectrum using Equation 3 (36),

\[
R_0^2 = (8.79 \cdot 10^{-23})/(\kappa^2 \eta^{-4} \Phi_D J)
\]  
(Eq. 3)

where the orientation factor \( \kappa^2 = 0.67 \) (assuming random orientation of the donor and acceptor), the refractive index of the buffer was measured as \( \eta = 1.33 \), and the overlap integral \( J \) was calculated by using PhotochemCAD software (http://www.photochemcad.com/pages/chemcad/chem-home.html)\(^3\) (39). The relative quantum yield (\( \Phi_{\text{rel}} \)) of CoA-488–labeled nhTMEM16 was determined to be 0.44. This number was obtained by comparison with a reference solution of fluorescein in 0.1 N NaOH (fluorescein has a quantum yield \( \Phi_F = 0.95 \)) using Equation 4 (36),

\[
\Phi_D = \Phi_D(G_D/G_F)(\eta_D^2/\eta_D^2)
\]  
(Eq. 4)

where \( G_D \) and \( G_F \) are the gradients of plots of integrated fluorescence intensity versus absorbance for CoA-488–labeled TMEM16 and the fluorescein standard, respectively, and \( \eta_D \) and \( \eta_F \) are the refractive indices of the corresponding solutions.

**Distance between the \( N \) terminus of one monomer and the \( C \) terminus of its partner by using FLIM–FRET measurements**

Purified MCP–TMEM16–ACP was doubly labeled as follows. The ACP tag was labeled with CoA-488 using ACP synthase as described above. The labeled protein was purified by gel filtration, and a portion was incubated with CoA-547 in the presence of SFP synthase to label the MCP tag. The CoA-488–labeled protein (donor) and CoA-488/CoA-547 (donor + acceptor)–labeled protein samples were diluted in Buffer D. Protein concentrations were determined by measuring absorbance at 280, 488, and 550 nm for protein, 488-dye, and 547-dye, respectively. Labeling efficiency (determined as described above; for the 547-dye we used \( e_{\text{dye}} = 150,000 \text{ M}^{-1}\text{cm}^{-1} \) at 555 nm) was ~80% for both MCP and ACP tags.

FLIM images were acquired on solutions of the donor only and donor + acceptor samples using digital frequency-domain fluorescence lifetime imaging on a FLIM/FFS laser-scanning microscope equipped with 488 nm ISS Albav5 at a base frequency of 20 MHz. A dichroic filter (530/43 nm) was used to collect the donor emission within a 50-µm pinhole onto a photomultiplier tube detector. FLIM data were collected using 60% of laser power and 100-µs pixel dwell time over 10 frames for CoA-488–labeled MCP–TMEM16–ACP (donor) and 60 frames CoA-488/CoA-547–labeled MCP–TMEM16–ACP (donor + acceptor) in a 20 × 20-µm image of 256 × 256 pixels. Each pixel is represented as a point in a phasor plot with \( S \) and \( G \) coordinates, i.e. a single exponential lifetime point is located on the semi-circle (37) defined by Equations 5 and 6.

\[
S = m \sin \varphi = \omega \tau/(1 + \omega^2 \tau^2)
\]  
(Eq. 5)

\[
G = m \cos \varphi = 1/(1 + \omega^2 \tau^2)
\]  
(Eq. 6)

FLIM was calibrated with 46 nm fluorescein in 50 mM Tris buffer at pH 8 (reference fluorescence lifetime of 4.04 ns) under similar conditions of protein samples for 10 frames. After the measurements of phase delays and modulation ratios of the emission of each image at a frequency of 40 MHz, the phasor position of all pixels was determined using Equations 5 and 6 (Fig. 5, A and B). The ratio of the donor lifetimes determined in the absence (\( \tau_F \)) and in the presence of the acceptor (\( \tau_{\text{DA}} \)) allows us to estimate the FRET efficiency (\( E_{\text{FRET}} \)) by using Equation 7 (38).

\[
E = 1 - \tau_{\text{DA}}/\tau_F
\]  
(Eq. 7)

Using \( E \) and \( R_0 \) values, the distance between the donor and acceptor can be calculated by Equation 8 (31).

\[
r = R_0/((1/E) - 1)^{1/6}
\]  
(Eq. 8)

**Orientations of TMEM16 in proteoliposomes**

The 488 dye is susceptible to chemical reduction by dithionite, resulting in loss of fluorescence. ACP-tagged nhTMEM16 constructs labeled via CoA-488 were reconstituted into vesicles consisting of three parts (3 POPE, 1 POPG) to 1 part 60% egg PC as described above. The reconstituted sample (20 µl, diluted to 2 ml in Buffer D) was placed in a cuvette, and its fluorescence was monitored over time (\( \lambda_{\text{ex}} = 492 \text{ nm} \) and \( \lambda_{\text{em}} = 520 \text{ nm} \)) in a fluorescence spectrometer. Dithionite (20 mM final concentration, from a 1 M stock solution freshly prepared in 0.5 M Tris-HCl, pH 10) was added to eliminate fluorescence of fluorophores accessible at the vesicle exterior. Triton X-100 (final concentration 0.1% (w/v)) was subsequently added to permeabilize the vesicles, enabling dithionite to reduce all dye molecules.


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**References**


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