Change in Conformation of Plasma Membrane Phospholipid Scramblase Induced by Occupancy of Its Ca^{2+} Binding Site†

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ABSTRACT: Phospholipid (PL) scramblase is a 35.1 kDa plasma membrane protein that mediates the accelerated transbilayer migration of plasma membrane PL in activated, injured, or apoptotic cells exposed to elevated intracellular Ca^{2+}. We recently identified a conserved segment in the PL scramblase polypeptide (residues Asp273 to Asp284) that is essential for its PL-mobilizing function and was presumed to contain the Ca^{2+} binding site of the protein (Zhou, Q., Sims, P. J., and Wiedmer, T. (1998) *Biochemistry 37*, 2356−2360). Whereas the sequence of this peptide segment resembles that of known Ca^{2+}-binding loops within EF-hand containing proteins, it is unusual in being a single such loop in the entire protein and in being closely spaced to the predicted transmembrane helix (Ala291-Gly309). To gain insight into how Ca^{2+} activates the PL-mobilizing function of PL scramblase, we analyzed conformational changes associated with occupancy of this putative Ca^{2+} binding site. In addition to activation by Ca^{2+}, the PL-mobilizing function of PL scramblase was found to be activated by other ions, with apparent affinities Tb^{3+}, $La^{3+} \gg Ca^{2+} > Mn^{2+} > Zn^{2+} > Sr^{2+} \gg Ba^{2+}$, Mg^{2+}. Evidence for coordinate binding of metal ion by the polypeptide was provided by resonance energy transfer from protein Trp to Tb^{3+}, which was competed by excess Ca^{2+}. Metal binding to PL scramblase was accompanied by increased right-angle light scattering and by a prominent change in circular dichroism, suggesting that coordinate binding of the metal ion induces a conformational change that includes self-aggregation of the polypeptide. Consistent with this interpretation, addition of Ca^{2+} was found to protect PL scramblase from proteolysis by trypsin both in detergent solution as well as in situ, within the erythrocyte membrane. Mutation in the segment Asp273-Asp284 reduced Tb^{3+} incorporation and attenuated the change in CD spectrum induced by bound metal ligand, confirming that this suspected EF-hand loopike segment of the polypeptide directly contributes to the Ca^{2+} binding site.

Plasma membrane phospholipids (PL)[1] are normally asymmetrically distributed, with phosphatidylcholine and sphingomyelin located primarily in the outer leaflet, and the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine, concentrated in the cytoplasmic leaflet of the membrane (*1*, *2*). In resting cells, the rate of spontaneous exchange of PL between plasma membrane leaflets is very slow. An increase in intracellular Ca^{2+} due to either cell activation, injury by complement, or apoptosis initiates a rapid bidirectional movement of plasma membrane PL between leaflets, collapsing the compositional asymmetry and resulting in *de novo* exposure of PS and phosphatidylethanolamine at the cell surface (*2*−*7*). The exposure of these aminoPL has been shown to promote cell surface assembly of key enzymes of the plasma coagulation and complement systems and has been implicated in phagocytic removal of injured or apoptotic cells by the reticuloendothelial system. This suggests that Ca^{2+}-induced redistribution of plasma membrane PL plays a central role in the regulation of coagulation and in cellular clearance mechanisms (*8*−*14*).

We recently reported the deduced protein sequence of PL scramblase, an integral membrane protein capable of mediating the Ca^{2+}-dependent transbilayer movement of PL, in a manner similar to that observed for plasma membrane PL exposed to Ca^{2+} at the endofacial surface. This 318 residue, predicted type 2 membrane protein, was initially isolated from human erythrocytes and subsequently shown to be expressed in platelets, lymphocytes, and a variety of other cells and tissues. When incorporated into lipid vesicles, PL scramblase induced rapid bidirectional movement of all PL between membrane leaflets in response to added Ca^{2+}, or to acidification (pH < 6.5) (*15*, *16*). Furthermore, when various human cell lines were compared, the level of expression of PL scramblase was found to correlate with the exposure of PS at the cell surface in response to an influx of Ca^{2+} into the cytosol (*17*). Transfection of a cell line deficient in endogenous PL scramblase with cDNA encoding the protein

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[1] Abbreviations: PL, phospholipid(s); RBC, red blood cell; PS, phosphatidylserine; NBD-PS, 1-oleoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine; OG, octyl D-glucoside; MBP, maltose binding protein; CD, circular dichroism.

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was shown to up regulate the transmembrane movement of plasma membrane PL in response to elevation of intracellular Ca^{2+} (*17*). This suggests that PL scramblase is the component of the plasma membrane that is responsible for the reorganization of cell surface PL under conditions of elevated cytosolic Ca^{2+}.

Analysis of the sequence conserved in both human and murine PL scramblase polypeptides revealed a single segment (Asp273-Asp284, in human) displaying similarity to known Ca^{2+}-binding EF-hand loop motifs. This single EF-hand-like loop found in PL scramblase is located in close proximity to the predicted transmembrane helix (Ala291-Gly309) of the protein (*18*). Mutation within this putative Ca^{2+}-binding site confirmed that residues contained within this loop were essential for normal protein function in promoting transbilayer movement of membrane PL (*18*). Here we report evidence that this segment in PL scramblase is indeed the Ca^{2+} binding site and that the incorporation of bound metal into this portion of the polypeptide induces a prominent conformational change in the protein that includes oligomerization, suggesting possible mechanisms by which Ca^{2+} binding to PL scramblase might accelerate transbilayer movement of plasma membrane PL.

EXPERIMENTAL PROCEDURES

Materials. Human erythrocytes were obtained from The Blood Center of Southeastern Wisconsin. Egg yolk phosphatidylcholine, brain PS, and 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine (NBD-PS) were obtained from Avanti Polar Lipids. *N*-Octyl-β-D-glucopyranoside, Glu-Gly-Arg chloromethyl ketone, zwittergent 3-12, and BRIJ 35 were from Calbiochem. Terbium(III) chloride, lanthanum chloride, and strontium chloride were obtained from Aldrich. Factor Xa was from Haematologic Technologies, and amylose resin, from New England Biolabs. Bio-Beads SM-2 adsorbent, Chelex 100 resin, and SDS were from BioRad. Buffers, salts, TPCK-treated bovine pancreatic trypsin (12 000 units/mg), and all other reagents were purchased from Sigma. Sodium dithionite ($Na_2S_2O_4$, Sigma) was freshly dissolved in 1 M Tris, pH 10, at a concentration of 1 M.

Human Erythrocyte PL Scramblase. Human erythrocyte ghost membranes and purified PL scramblase isolated from erythrocyte ghost membranes were prepared essentially as described previously (*15*, *19*).

Recombinant PL Scramblase. Human PL scramblase and the Asp275→Ala mutant of human PL scramblase were each expressed as MBP fusion proteins and purified from *Escherichia coli* as previously described (*18*). After elution from the amylose resin with 10 mM maltose in 20 mM Tris, 200 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.4, the peak of protein, observed by A_{280nm}, was pooled and centrifuged at 155000*g* for 30 min at 4 °C. Total protein in the supernatant was determined by A_{280nm} and 5 mM zwittergent 3-12 final concentration was added. The purified PL scramblase−MBP fusion protein was digested with factor Xa at 1:100 w/w enzyme/protein at 23 °C for 24 h. The presence of zwittergent 3-12 during digestion preserves the native molecular size of PL scramblase and prevents nonspecific degradation observed when this digest was performed with other detergents (data not shown). Digestion was monitored by SDS−PAGE using 12.5% acrylamide Phast gel and visualized by staining with silver (Phast System, Pharmacia Biotech Inc.). After digest, factor Xa was inhibited with 100 μM Glu-Gly-Arg chloromethyl ketone (30 min, 23 °C). The sample was diluted 5-fold with 20 mM Tris, 0.1 mM EGTA, and 5 mM zwittergent 3-12, pH 7.4, and loaded (1 mL/min) onto a 0.5- × 5-cm Mono Q HR column (Pharmacia) equilibrated in the same buffer. The column was then washed with 20 column volumes of the same buffer, substituting 0.025% BRIJ 35 for zwittergent 3-12. Protein was eluted (1 mL/min) by an initial step to 150 mM NaCl, followed by a step to 1 M NaCl in the same buffer, collecting 1-mL fractions. MBP was observed to elute in 150 mM NaCl, whereas PL scramblase eluted in 1 M NaCl. The recombinant PL scramblase was >95% pure as determined by SDS−PAGE and was either used immediately or stored frozen at −80 °C.

Reconstitution and Functional Assay in Proteoliposomes. Reconstitution and activity assay of PL scramblase was performed as previously described (*15*, *16*, *18*, *19*). In brief, erythrocyte PL scramblase was reconstituted in liposomes containing exogenous PL (10% PS, 90% phosphatidylcholine) at 4 mg/mL total PL by dialysis against 100 mM Tris, 100 mM KCl, and 0.1 mM EGTA, pH 7.4, containing 1 g/L Bio-Beads SM-2 adsorbent. The resulting proteoliposomes were recovered and diluted (0.4 mg/mL PL) in 100 mM Tris, 100 mM KCl, and 0.1 mM EGTA, pH 7.4, and 0.25 mol % NBD-PS was added and allowed to incorporate into the outer membrane leaflet for 5 min at 23 °C. The samples were then incubated (3 h, 37 °C) in the presence of Ca^{2+} or other metal to be tested, before dilution into 25 volumes of 100 mM Tris and 100 mM KCl, pH 7.4, containing 4 mM EGTA. The metal ion dependent movement of NBD-PS from outer to inner leaflet during the 3 h incubation at 37 °C was deduced from the resulting sequestration of the fluorescent NBD moiety from the membrane-impermeant quencher, sodium dithionite, added externally (*15*). Ionized [Ca^{2+}] was calculated using FreeCal version 4.0 software (generously provided by Dr. Lawrence F. Brass, University of Pennsylvania, Philadelphia, PA). Other ionized [metals] were calculated on the basis of the free [Ca^{2+}].

Fluorescence Spectroscopy. Purified recombinant PL scramblase (wild-type or Asp275→Ala mutant) was dialyzed against 100 mM MES and 0.025% BRIJ 35, pH 6.0 (for assay with Tb^{3+}), or 100 mM Tris and 0.025% BRIJ 35, pH 7.4 (for assay with Ca^{2+}), to which Chelex 100 resin was added. In the case of Tb^{3+}, measurements were made at acid pH to minimize precipitation. Stock solutions of metal salts (1 M $CaCl_2$, 10 mM $TbCl_3$) were freshly prepared in the respective dialysis buffer and all samples and solutions were filtered through a 0.45 μm low protein binding membrane. Protein was diluted into dialysis buffer to final concentrations of 1−10 μM. All fluorescence measurements were performed using an SLM Aminco 8100 spectrofluorometer with sample compartment maintained at 23 °C. Excitation and emission band passes were set at 1 and 4 nm, respectively. Tryptophan was excited at 295 nm and emission spectra were recorded at 2 nm intervals between 320 and 600 nm using 2 s integration. Spectra for a buffer blank and protein sample at each Tb^{3+} and/or Ca^{2+} concentration were recorded. For resonance energy transfer to Tb^{3+},

emission specific to the protein-bound Tb^{3+} at wavelengths of 492 and 546 nm was evaluated, with correction of the appropriate buffer blank omitting protein. Due to light scattering from the PL vesicles, we were not able to resolve the protein emission spectra using membrane-incorporated PL scramblase.

Circular Dichroism Spectroscopy. Purified wild-type or mutant recombinant PL scramblase was prepared for circular dichroism (CD) spectroscopy by dialysis against 10 mM Hepes, 0.01 mM EGTA, and 0.025% BRIJ 35, pH 6.8. Measurements were performed at pH 6.8, to accommodate solubilities of $TbCl_3$ and $LaCl_3$, while minimizing potential acid-induced conformational changes in PL scramblase (*15*, *16*). Protein samples were filtered through a 0.45 μm low protein binding membrane and concentrations were determined as described below. Spectra were obtained at 23 °C with recombinant PL scramblase suspended in either EGTA, $CaCl_2$, $LaCl_3$, or $TbCl_3$ using a Jasco J-710 CD spectrometer with a 0.1-cm path length quartz cell. Spectra represent the average of four accumulations recorded between 200 and 250 nm, with readings at 1-nm intervals and integration time of 2 s. All spectra were corrected for contributions from the buffer. Due to significantly increased absorption of the buffer blank, scans below 200 nm were not performed.

Tryptic Digest. Erythrocyte PL scramblase was dialyzed into digest buffer (50 mM Tris, 25 mM OG, 1 mM dithiothreitol, 100 mM NaCl, and 0.1 mM EGTA, pH 7.7). Matched samples each containing 45-μg of PL scramblase and either 0 or 2 mM Ca^{2+} were first incubated for 5 min at 37 °C. Predigest ($t = 0$) samples were removed and then trypsin added at 1:25 w/w enzyme/protein. Proteolysis was allowed to proceed at 37 °C, and aliquots were taken at various time points and denatured by heating in SDS−PAGE sample buffer containing 10% 2-mercaptoethanol. Samples were stored frozen at −20 °C prior to analysis. Tryptic digest of PL scramblase present in the erythrocyte membrane was performed using white erythrocyte ghost membranes that were washed and suspended at 3 mg/mL total protein in digest buffer (*above*) omitting OG. Matched samples each containing 400 μg of total protein and either 0 or 2 mM Ca^{2+} were first incubated for 5 min at 37 °C before addition of either 0 or 4 mM EGTA. After the samples were cooled to to 23 °C, predigest samples ($t = 0$) were removed, and trypsin was added at 1:100 w/w enzyme/protein. The digest was allowed to proceed at 23 °C with continuous agitation of the membrane suspension, and aliquots were taken at various time points and stopped by heating in SDS sample buffer as described above.

Western Blotting. Samples from the tryptic digests were resolved by SDS−PAGE on 12.5% homogeneous Phast gels and electroblotted onto 0.45-μm poly(vinylidene difluoride) membranes for Western analysis (Phast System, Pharmacia Biotech Inc.). Western blotting of PL scramblase was performed using affinity purified antibody against the C-terminal residues of the polypeptide as previously described (*16*, *17*).

Protein Concentration. Concentration of PL scramblase (wild-type and Asp275→Ala mutant) was determined from absorbance at 280 nm, using the calculated extinction coefficient of 39 000 M^{-1} cm^{-1} (*16*, *18*).

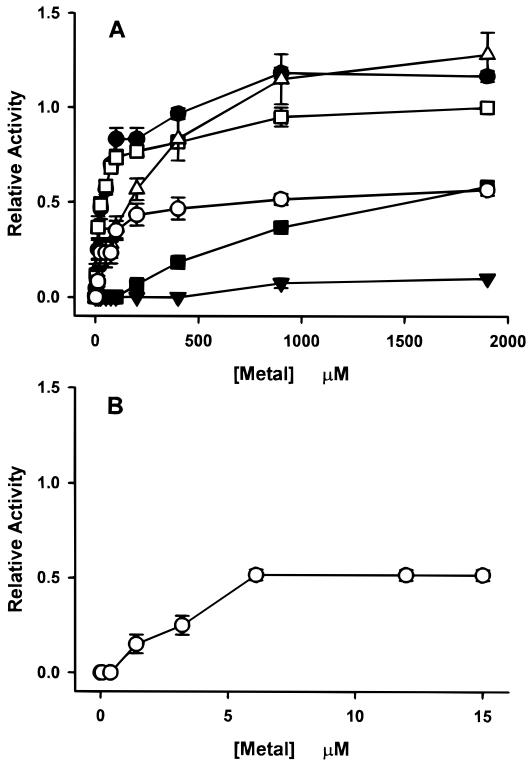

FIGURE 1: Ion selectivity for the activation of PL scramblase. The transbilayer movement of NBD-PS in proteoliposomes reconstituted with erythrocyte PL scramblase plotted for various metal ions at concentrations indicated by abscissa. Movement of NBD-PS from outer to inner membrane leaflet was measured after 3 h incubation at 37 °C with correction for nonspecific transbilayer migration of probe in vesicles omitting PL scramblase. Distribution of NBD-PS between membrane leaflets was determined by dithionite quenching of NBD fluorescence (see Experimental Procedures). All data are normalized to activity measured at 2 mM [Ca^{2+}]. Error bars denote mean ± SD of triplicate determinations performed on a single vesicle preparation incubated at each ion concentration indicated. All data derive from a single experiment and are representative of three similar experiments that were performed on separate days with freshly reconstituted PL scramblase. Panel A: Relative activity plotted as function of concentration of Ca^{2+} (□), Mn^{2+} (●), Sr^{2+} (△), Zn^{2+} (○), Ba^{2+} (■), or Mg^{2+} (▼). Panel B: Relative activity plotted as function of concentration of Tb^{3+} . Note difference in scale on abscissa.

Table 1: Ion Selectivity of PL Scramblase

metal	EC_{50} (μM)[a]
Ca^{2+}	28.7 ± 4.1
Mn^{2+}	44.9 ± 6.1
Zn^{2+}	78.9 ± 13.5
Sr^{2+}	308 ± 28.6
Ba^{2+}	2783 ± 439
Mg^{2+}	7135 ± 4446
	mean ± SE ($n = 3$)

[a] Concentration at which half-maximal PL scramblase activity was observed.

RESULTS AND DISCUSSION

Metal Selectivity. In addition to its known activation by Ca^{2+}, the PL-mobilizing function of PL scramblase was induced by several other divalent metal ions, with relative apparent affinities among these ions generally conforming to that reported for other known Ca^{2+} binding proteins (Figure 1 and Table 1) (*20*−*22*). The apparent affinity of this protein for activation by Ca^{2+} suggests a $K_d > 10^{-5}$,

which is consistent with the substantial elevation of intracellular Ca^{2+} that is required in order to observe accelerated movement of PL between plasma membrane leaflets in erythrocytes, platelets, and other cells (refs *15*, *18*, *19*) and references therein). Due presumably to this relatively low affinity for the ion, we have not been able to demonstrate specific binding of $^{45}Ca^{2+}$ to purified PL scramblase (data not shown). In addition to activation by Ca^{2+} and similar divalent metal ions, the PL-mobilizing function of PL scramblase was also activated by lanthanide metals, including Tb^{3+} (Figure 1, panel B). Whereas the data of Figure 1 suggest that the apparent affinity of PL scramblase for Tb^{3+} is considerably higher than it is for Ca^{2+}, it should be noted that we were unable to monitor transbilayer movement of the NBD-PS at Tb^{3+} concentrations above 20 μM, due to leakage of the anionic quencher dithionite across the proteoliposome membrane under these conditions (see Experimental Procedures). Similar results to that shown for Tb^{3+} were obtained when La^{3+} was used to activate PL-mobilizing function of membrane-incorporated PL scramblase (data not shown).

Tb^{3+} Luminescence Spectroscopy. As the data of Figure 1 suggested that Tb^{3+} could substitute for Ca^{2+} in inducing the active conformation of PL scramblase in the liposome membrane, we explored the possibility to detect binding of this ion to recombinant PL scramblase polypeptide in detergent solution, using fluorescence energy transfer from tryptophan residues to protein-bound Tb^{3+} (Figures 2 and 3). When excited at 295 nm in the presence of PL scramblase, luminescence from Tb^{3+} at its emission wavelengths of 492 and 546 nm increased with both Tb^{3+} and protein concentration in a manner consistent with saturable binding of the ion to a site in the protein where energy transfer from tryptophan(s) might occur. In addition to the increased emission from bound Tb^{3+}, we observed a distinct increase in right-angle light scatter of the protein solution (measured at the first harmonic of the excitation wavelength of 295 nm; Figure 2, panel A). The detected increase in light scatter distinctly lagged the increase in energy transfer (monitored by Tb^{3+} luminescence at either 492 or 546 nm), suggesting that protein aggregation occurred secondarily to the incorporation of metal ligand into the polypeptide. A distinct increase in right angle light scattering from PL scramblase was also observed upon addition of Ca^{2+}, suggesting that Ca^{2+} binding is accompanied by self-aggregation of the protein (see below). The luminescence from protein-bound Tb^{3+} was also partially competed by Ca^{2+}, implying that both ions competed for binding to the same segment of the polypeptide (Figure 3). Failure of Ca^{2+} to completely inhibit Tb^{3+} binding may reflect irreversible binding of Tb^{3+} within protein aggregates or Tb^{3+} binding to other segments of PL scramblase in addition to the actual Ca^{2+} binding site. Also to be noted, the apparent affinities for incorporation of Ca^{2+} and Tb^{3+} into recombinant PL scramblase in detergent solution (Figures 2 and 3) were each distinctly lower than would be implied by the metal-dependent activation of the PL-mobilizing function of native erythrocyte PL scramblase reconstituted in liposome membranes (Figure 1). This raises the possibility that (1) the higher apparent metal affinity of PL scramblase reconstituted in the proteoliposome versus in detergent solution reflects coordinate chelation of the ion by residues contributed by more than a single membrane-inserted polypeptide or (2) that the metal affinity of the bacterially expressed PL scramblase is intrinsically lower than that of PL scramblase isolated from human erythrocyte, due either to improper folding of the

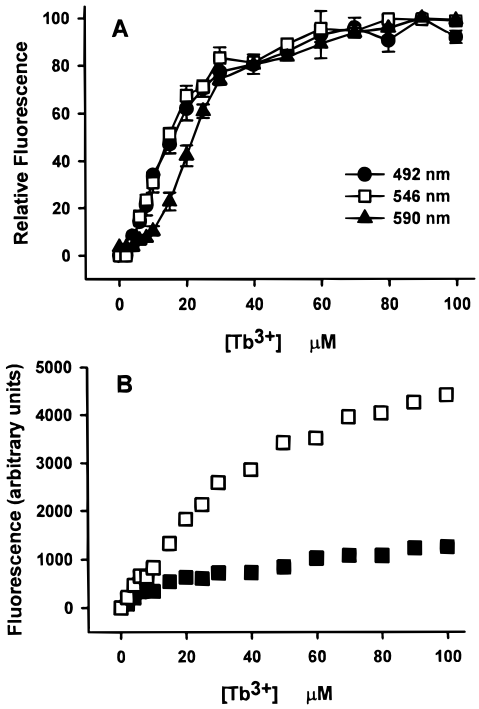

FIGURE 2: Terbium luminescence spectroscopy of recombinant PL scramblase. Panel A: Luminescence from protein-bound Tb^{3+} at emission wavelengths of 492 (●) and 546 nm (□) was measured at pH 6.0 for 1 μM recombinant PL scramblase incubated in the presence of the various Tb^{3+} concentrations indicated on abscissa. Excitation was at 295 nm. Also shown is light scattering (▲) measured at the first harmonic (590 nm) of the excitation wavelength. All data are corrected for background emission recorded at each Tb^{3+} concentration, omitting protein, and are normalized to maximum signal recorded at each wavelength. Error bars denote mean ± SD ($n = 3$). Panel B: Emission from protein-bound Tb^{3+} at 546 nm is compared for recombinant PL scramblase at protein concentrations of 1μM (closed symbols) and 10 μM (open symbols). Similar results were obtained when emission at 492 nm was analyzed (not shown). Data of single experiment, representative of three so performed.

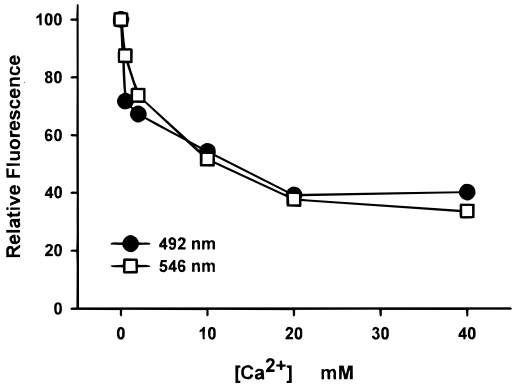

FIGURE 3: Tb^{3+} incorporation into PL scramblase competed by Ca^{2+}. Recombinant PL scramblase was diluted to 2 μM protein in the presence of 20 μM Tb^{3+} and the concentration of Ca^{2+} indicated by abscissa. After 5 min of incubation at room temperature, emission spectra were obtained as indicated for Figure 2. Emission from protein-bound Tb^{3+} at either 492 (●) or 546 nm (□) is plotted at each Ca^{2+} concentration, with normalization to zero Ca^{2+}. Data of single experiment, representative of two so performed.

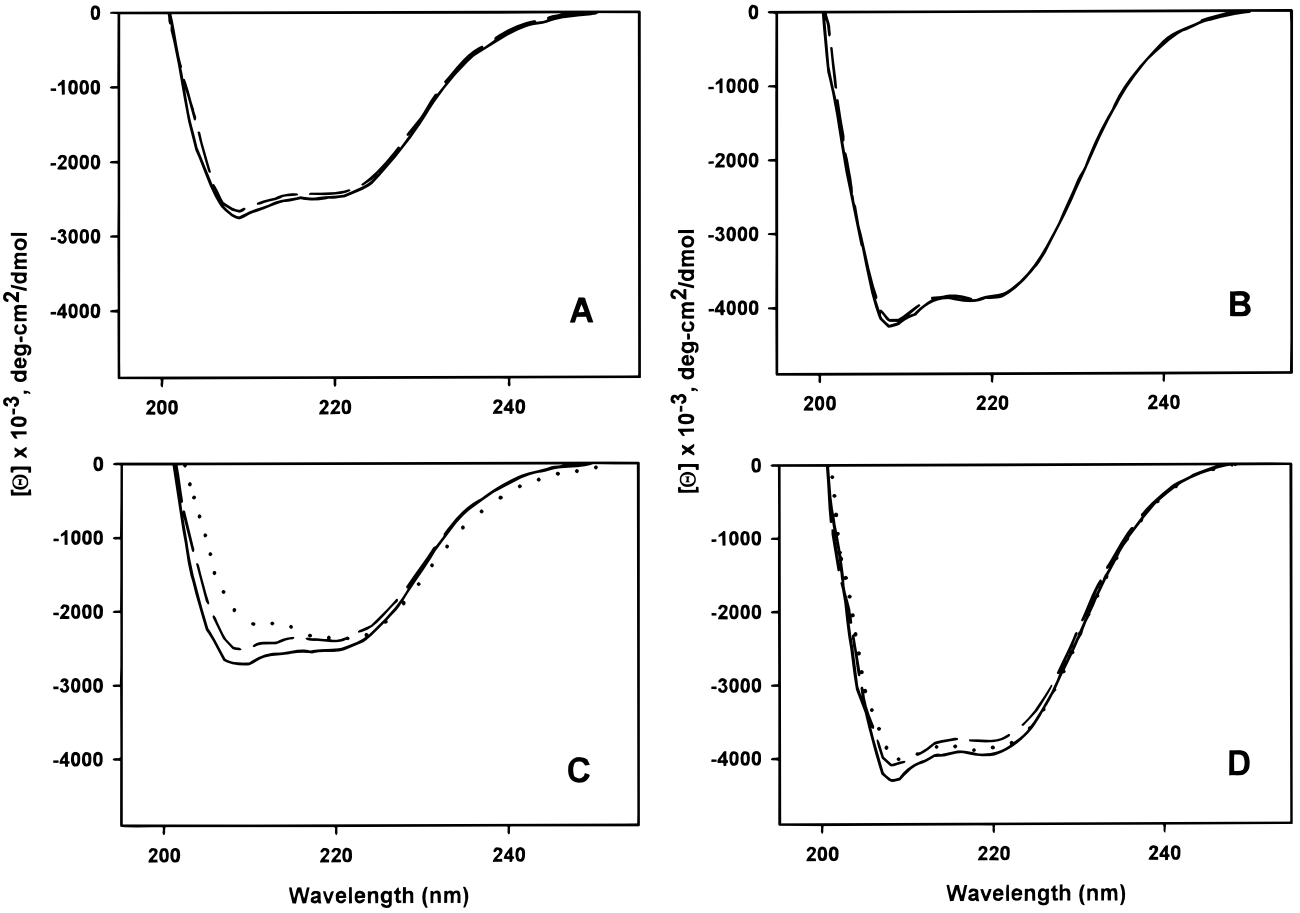

FIGURE 4: Circular dichroism spectroscopy of recombinant PL scramblase. CD spectra of wild-type PL scramblase and PL scramblase containing Asp275→Ala mutation within the putative Ca^{2+}-binding loop. All spectra were obtained with 9 μM protein at room temp (pH 6.8). Spectra are corrected for background contributions from the buffer, omitting protein. In each panel, molar ellipticity (ordinate) is plotted against wavelength (abscissa). Each spectrum shown represents the average of four scans, without smoothing. Panel A: CD spectra of wild-type PL scramblase in the absence (solid line) and presence of 2 mM Ca^{2+} (dashed line).The small change in the CD spectrum when Ca^{2+} was present (dashed line, Panel A) is typical of that observed in at least three separate experiments that were performed at various protein concentrations (data not shown). Panel B: CD spectra of mutant Asp275→Ala PL scramblase in the absence (solid line) and presence of 2 mM Ca^{2+} (dashed line). Panel C: CD spectra of wild-type PL scramblase in the absence (solid line) and presence of either 40 μM La^{3+} (dashed line) or 60 μM La^{3+} (dotted line). Panel D: CD spectra of mutant Asp275→Ala PL scramblase in the absence (solid line) and presence of either 40 μM La^{3+} (dashed line) or 60 μM La^{3+} (dotted line). Spectra obtained by substituting Tb^{3+} for La^{3+} were similar to those shown for La^{3+} (data not shown). Data of single experiment, representative of two so performed.

bacterial-derived protein or to a potential posttranslational modification of the polypeptide in mammalian cells affecting the metal binding site (see below). In this context, it is of interest to note that (1) by contrast to conventional Ca^{2+} binding proteins containing multiple EF-hand motifs, the sequence of PL scramblase reveals only a single potential Ca^{2+}-binding loop (Asp273-Asp285), predicting at best very low affinity Ca^{2+} binding to the isolated polypeptide (*18*, *21*). On the other hand, alignment of multiple PL scramblase in a lipid membrane might allow Ca^{2+} to bridge residues of the Asp273-Asp285 loops contributed by two or more polypeptides; (2) when PL scramblase activity was directly compared for proteoliposmes reconstituted with the bacterial-expressed human protein versus PL scramblase isolated from human erythrocyte, a slightly lower apparent affinity for Ca^{2+} was noted for the recombinant protein (half-maximal activation at ~100 μM Ca^{2+} (recombinant PL scramblase) versus ~20−40μM (erythrocyte PL scramblase; see refs *16*, and *18* and Table 1), but this difference is not sufficient to account for the nearly 10-fold difference in apparent affinities observed for the interaction of either Ca^{2+} or Tb^{3+} with the detergent-solubilized versus membrane-incorporated protein.

Conformational Changes Associated with Occupancy of the Ca^{2+} Binding Site. As noted above, Tb^{3+} luminescence was accompanied by increase in protein light scatter (cf. Figure 2), and we observed a similar increase in Rayleigh light scatter with addition of Ca^{2+} to PL scramblase (data not shown), implying that the bound metal ligand induces oligomerization of the protein, either by cross-linking PL scramblase monomers or by inducing a conformational change in the polypeptide that in turn promotes self-association. Evidence that PL scramblase undergoes conformational rearrangement upon binding a metal ligand was also suggested by changes in the CD spectrum of polypeptide upon addition of either Ca^{2+} or La^{3+} (Figure 4). Consistent with the interpretation that the observed change in protein ellipticity specifically reflects interaction of the metal ion at the single EF-hand loop segment in PL scramblase, we observed that mutation within this segment of the polypeptide abrogated most of the spectral response induced by either Ca^{2+} or La^{3+} (Figure 4). The marked differences we observe

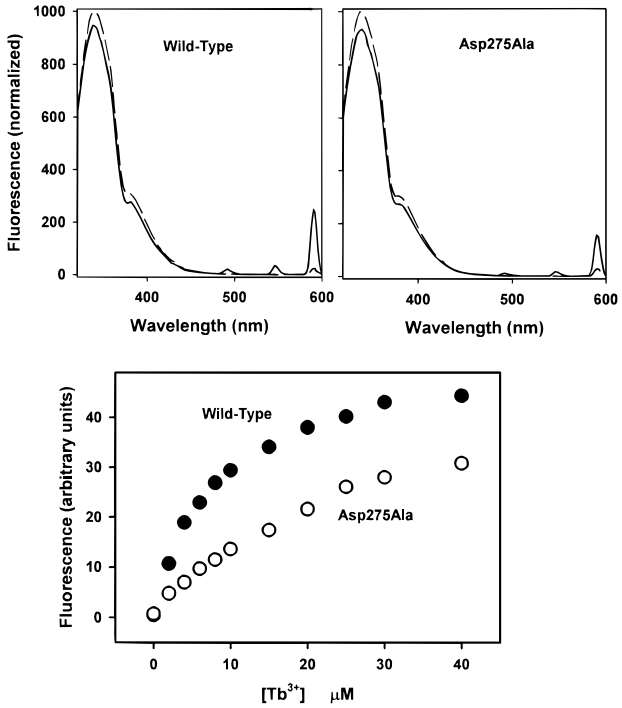

FIGURE 5: Terbium luminescence spectroscopy of wild-type and Asp275→Ala PL scramblase. Fluorescence emission spectra were obtained for 1 μM recombinant PL scramblase (wild-type or Asp275→Ala mutant) equilibrated with Tb^{3+} at concentrations indicated. Excitation was at 295 nm. Luminescence from protein-bound Tb^{3+} was observed at emission wavelengths 492 and 546 nm as indicated, and right angle light scatter at the first harmonic (590 nm) of the exciting wavelength. All data are corrected for background emission recorded at each Tb^{3+} concentration, omitting protein, and are normalized to max fluorescence = 1000 for protein in the absence of Tb^{3+}, respectively (see Experimental Procedures). Data of single experiment, representative of two so performed. Upper panels: Emission spectra of wild-type (left-hand panel) and Asp275→Ala mutant (right-hand panel) were obtained in the presence of 0 (dashed trace) or 20 μM (solid trace) Tb^{3+}. Lower panel: Emission intensity at 546 nm of wild-type (●) and mutant (○) PL scramblase, plotted as function of $[Tb^{3+}]$.

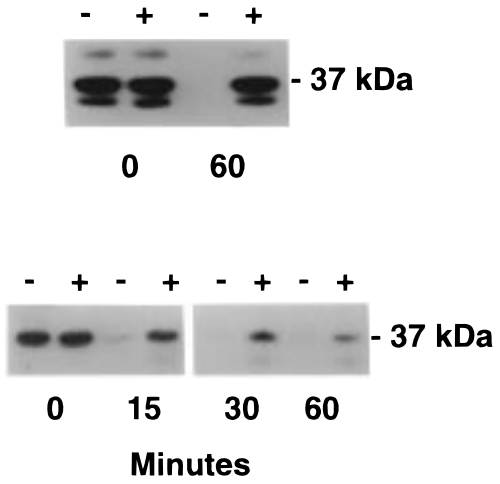

FIGURE 6: Protease digest PL scramblase was subjected to digest by trypsin in the absence (−) and presence (+) of 2 mM Ca^{2+}. Aliquots at various time points were resolved by SDS−PAGE and developed by Western blotting with antibody against the carboxyl-terminal peptide of PL scramblase (see Experimental Procedures). Times indicate minutes after addition of trypsin. Upper panel: Tryptic digest of purified human erythrocyte PL scramblase performed in 25 mM OG. Bottom panel: In situ tryptic digest of PL scramblase contained in erythrocyte ghost membranes, performed in the absence of detergent. Data of single experiment, representative of three so performed.

in protein ellipticity induced by La^{3+} versus Ca^{2+} is quite similar to that previously reported for the interaction of these ions with single EF-hand loop peptides derived from parvalbumin and other isolated EF-hand-containing peptides (*23*, *24*). In those cases, a marked increase in peptide ellipticity induced by La^{3+} (versus Ca^{2+}) was directly attributed to the higher charge density of the lanthanide metals which enables these ions to induce changes in peptide secondary structure and to fully occupy a single metal-binding loop that, in the case of Ca^{2+}, is incomplete and only partially condenses about the ion (*23*, *24*). To be noted, measured ellipticity can be affected by protein oligomerization as well as by the differential rotary dispersion of light scattered from large protein aggregates (*25*). Thus the spectral changes observed in Figure 4 are likely to reflect combined effects of metal−ligand induced changes in protein secondary structure, self-aggregation of the polypeptide, and a contribution of light scatter. Accordingly, a quantitative analysis of the CD spectra of PL scramblase in terms of actual secondary structure of the polypeptide was not performed.

In addition to substantially abrogating the metal−ligand induced change in circular dichroism, mutation at Asp275 also appeared to affect the CD spectrum of the polypeptide, in absence of added metal (cf. solid lines in Figure 4, panels A and C versus panels B and D). The apparent increase in the molar ellipticity of the Ala275 mutant versus wild-type may reflect actual differences in secondary structure or experimental error in matching protein concentrations of the recombinant proteins. An apparent reduction in metal binding to the Ala275 mutant PL scramblase (versus wild-type) was also detected when luminescence from protein-bound Tb^{3+} was measured (Figure 5). Taken together with the data of Figure 4, these results suggest that Asp275 participates in coordinating metal ligands and to the induced rearrangement of the PL scramblase polypeptide in the metal ligand-bound state, which is consistent with the observed reduction in membrane PL scramblase activity that accompanied mutation in this segment of the polypeptide (*18*).

The Ca^{2+}-induced change in detergent-solubilized PL scramblase mimics that of PL scramblase in the erythrocyte membrane. The indication by light scattering that occupancy of the Ca^{2+} binding site in PL scramblase is accompanied by aggregation implied that the protein undergoes a conformational change in the presence of Ca^{2+} which includes self-association into an oligomer, and which we assume is related to the observed activation of the PL mobilizing function of this protein when Ca^{2+} is increased at the endofacial surface of the plasma membrane. In other proteins known to contain Ca^{2+} binding site(s), the conformational change induced by the bound ion can result in diminished access of trypsin and other proteases to the peptide backbone, which is reflected by relative protection of the protein when proteolysis is performed in the presence of Ca^{2+} (*26*, *27*). As shown in Figure 6, upper panel, addition of Ca^{2+} to purified PL scramblase in detergent solution was found to substantially protect the protein from degradation by trypsin. This capacity of Ca^{2+} to inhibit access of trypsin to the peptide backbone of PL scramblase was also observed when proteolysis was performed using intact erythrocyte ghost membranes suspended in the absence of detergent (Figure

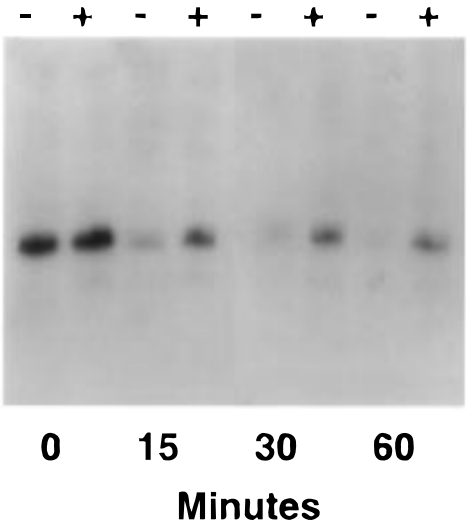

FIGURE 7: Irreversibility of Ca^{2+}-induced change in PL scramblase. In situ tryptic digest of PL scramblase in erythrocyte ghost membranes was performed as described in Figure 6, except membranes were incubated in the presence (+) or absence (−) of 2mM Ca^{2+} and then 4 mM EGTA was added to all samples before initiating digest with trypsin. Data of a single experiment that is representative of results obtained in a similar experiment.

6, lower panel). As had been observed for the optical changes associated with the binding of Tb^{3+} to PL scramblase (above), the effect of Ca^{2+} on the tryptic sensitivity of PL scramblase appeared irreversible, as the relative resistance to proteolysis that was conferred by Ca^{2+} persisted with subsequent addition of EGTA (Figure 7). These data confirm that Ca^{2+} binding to native PL scramblase projecting from the endofacial surface of the erythrocyte membrane induces a change within the protein affecting the conformation of the polypeptide and/or its state of aggregation, and that this induced change in the endogenous membrane protein in situ resembles that observed when Ca^{2+} interacts with the isolated protein in detergent solution. It remains to be determined how the conformational change(s) that arise in the membrane-inserted PL scramblase polypeptide—including potential cross-linking through a Ca^{2+} bridge—actually serves to accelerate the movement of membrane PL between the inner and outer leaflets.

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