Isolation of an Erythrocyte Membrane Protein that Mediates Ca^{2+}-dependent Transbilayer Movement of Phospholipid*

(Received for publication, March 22, 1996, and in revised form, May 6, 1996)

François Bassé, James G. Stout, Peter J. Sims, and Therese Wiedmer‡
From the Blood Research Institute of The Blood Center of Southeastern Wisconsin, Milwaukee, Wisconsin 53201

Elevation of intracellular Ca^{2+} in erythrocytes, platelets, and other cells initiates rapid redistribution of plasma membrane phospholipids (PL) between inner and outer leaflets, collapsing the normal asymmetric distribution. Consequently, phosphatidylserine and other lipids normally sequestered to the inner leaflet become exposed at the cell surface. This Ca^{2+}-induced mobilization of phosphatidylserine to the surface of activated, injured, or apoptotic cells confers a procoagulant property to the plasma membrane, which promotes fibrin clotting and provides a signal for cell removal by the reticuloendothelial system. To identify the constituent of the membrane that mediates this Ca^{2+}-dependent "PL scramblase" activity, we undertook purification and reconstitution of membrane component(s) with this activity from detergent extracts of erythrocyte ghosts depleted of cytoskeleton. Active fractions were identified by their capacity to mediate the Ca^{2+}-dependent redistribution of 7-nitrobenz-2-oxa-1,3-diazol-4-yl-labeled PL between leaflets of reconstituted proteoliposomes. This PL scramblase activity co-eluted through multiple chromatographic steps with a single polypeptide of ~37 kDa, which was purified to apparent homogeneity as resolved by silver staining. The activity associated with this protein band was inactivated by trypsin. The isolated protein reconstituted in proteoliposomes mediated nonselective, bidirectional transport of 7-nitrobenz-2-oxa-1,3-diazol-4-yl-PL between membrane leaflets, with half-maximal activation between 20 and 60 μM Ca^{2+} (saturation >100 μM), mimicking the Ca^{2+}-dependent transbilayer lipid movement intrinsic to the erythrocyte membrane.

The plasma membrane phospholipids (PL) of erythrocytes, platelets, and other cells are normally asymmetrically distributed; amino phospholipids, including phosphatidylserine (PS) and phosphatidylethanolamine (PE), reside almost exclusively in the inner membrane leaflet, whereas the outer leaflet is enriched in neutral polar PL, including phosphatidycholine (PC) and sphingomyelin (1, 2). Maintenance of this PL asymmetry arises principally through the activity of an amino phospholipid translocase, a putative Mg^{2+}-dependent ATPase that transports PS and PE, but not PC, from outer to inner membrane leaflet (2, 3). Upon an increase in intracellular Ca^{2+}, these lipids undergo rapid redistribution between the two membrane leaflets, ultimately resulting in a loss of this initial transbilayer asymmetry and de novo exposure of PS at the cell surface (1, 4–7). There is now abundant evidence that cell surface-exposed PS provides binding sites to promote assembly of factor VIIa/IXa (tenase) and factor Va/Xa (prothrombinase) enzyme complexes, contributing to the procoagulant activity expressed by activated or injured platelets, erythrocytes, and vascular endothelial cells (1, 8, 9). Surface-exposed PS has also been implicated as a signal for removal of damaged or aged cells through the reticuloendothelial system and is observed in cells undergoing apoptosis (10).

The mechanism by which amino phospholipids residing in the inner plasma membrane leaflet become surface-exposed is poorly understood. Transbilayer redistribution of membrane PL is activated by an increase in cytosolic Ca^{2+} and appears to be bidirectional and nonselective among polar PL according to some studies (4, 6, 11, 12), vectorial and PS and PE-specific according to others (7, 13). A requirement for ATP in this process is controversial (14, 15). Although accelerated transbilayer movement of plasma membrane PL was initially attributed to a spontaneous collapse of plasma membrane PL asymmetry due to Ca^{2+} inactivation of amino phospholipid translocase, this possibility is precluded by the inherently slow rate of transbilayer migration of PL, as well as by the failure of vanadate and other translocase inhibitors to mimic the PL scramblase activity evoked by intracellular Ca^{2+} (8). Furthermore, we have shown that blood cells from a patient with Scott syndrome, a congenital disorder associated with a selective defect in PL scramblase activity, exhibit normal amino phospholipid scramblase (16). Several studies, including our own (5), demonstrated a correlation between Ca^{2+}-induced plasma membrane vesiculation and PL scrambling, and we proposed that the membrane fusion event required for formation of these vesicles might underlie loss of PL asymmetry. Nevertheless, there is now evidence that surface exposure of PS can occur even when plasma membrane vesiculation is prevented (7). In other studies, inhibitors of enzymes that are known to be regulated by Ca^{2+}, such as calpain and transglutaminase, have been reported to affect expression of platelet procoagulant activity; however, a direct causal relationship between these enzyme activities and PL scrambling has not clearly been demonstrated, and PL scramblase activity persists in membrane vesicles devoid of cytosol (7, 12, 17, 18).

Recently, Sulpine and co-workers proposed a complex of Ca^{2+}-plus phosphatidylinositol 4,5-bisphosphate (PIP$_2$) as the initiating event in redistribution of plasma membrane PL (19). Evidence against a role for PIP$_2$ in Ca^{2+}-dependent membrane
PL scramblase was subsequently reported (16), and Williamson et al. (11) observed an inhibition of PL scramblase in platelets treated with the sulphydryl reagent pyridyl dithioethylamine, suggesting involvement of a protein. Thus, although a number of investigations have elucidated functional characteristics of the process of surface exposure of PS accompanying cell activation, the molecular identity of the membrane component(s) responsible for mediating this Ca\(^{2+}\)-induced redistribution of membrane PL remains unresolved.

Here we report purification and characterization of a membrane protein from human erythrocytes that promotes a Ca\(^{2+}\)-dependent transbilayer redistribution of PS and PC with properties similar to the PL scramblase activity that is evoked upon elevation of Ca\(^{2+}\) in the cytosol of erythrocytes and other cells.

**Experimental Procedures**

**Materials—** Egg yolk phosphatidylcholine (PC), brain phosphatidylserine (PS), 1-palmitoyl-2-oleoyl phosphatidic acid, 1-oleoyl-2-16 (7-nitrobenz-2-oxa-1,3-diazol-4-yl)aminocaproyl-sn-glycerol-3-phosphocholine (NB-PC), and 1-oleoyl-2-

**PL Scramblase Isolation—** Human erythrocytes were obtained from The Blood Center of Southeastern Wisconsin. Typically, one unit of packed erythrocytes was pelleted at 3,400 × g for 10 min at 4°C, and the cells were washed three times in cold 3.3 mM barbital, 150 mM NaCl, 0.02% NaN\(_3\), pH 7.4. The washed erythrocytes were lysed in 10 volumes of ice-cold 5 mM Na\(_2\)HPO\(_4\), 1 mM EDTA, 0.02% NaN\(_3\), 1 mM phenylmethylsulfonyl fluoride, pH 7.4, for 1 h at 4°C. Erythrocyte membranes were washed free of hemoglobin in this buffer by pelleting at 18,600 × g for 30 min at 4°C for three wash cycles and solubilized at a protein concentration of 35 mg/ml in a suspension of 0.1 M NaCl, 500 mM glucose, 100 mM EGTA, pH 7.0, for 10 min at 37°C. The solution was subsequently filtered through a 0.45-

**Trypsinization—** Samples containing active PL scramblase were incubated for 3 h at 37°C in the presence of 2 μg/ml trypsin in 100 mM Tris, 0.1 mM EDTA, 200 mM NaCl, pH 8.4. The reaction was stopped by addition of 1 μg/ml diisopropylfluorophosphate. Samples were incubated in the absence of trypsin or in the presence of trypsin plus 4 μg/ml soybean trypsin inhibitor served as control. Residual PL scramblase activity of each sample was then determined following reconstitution into NBD-PS outside-labeled proteoliposomes. Residual PL scramblase activity in trypsin-treated samples was normalized to that observed for identically matched samples incubated without trypsin.

**Preparation of Erythrocyte IOVs—** Erythrocyte inside-out vesicles (IOVs) were prepared essentially by methods described by Steck and Kant (21) with minor modifications. Assays for acetylcholinesterase (outside) and glycyldihydroxy-3-phosphate dehydrogenase accessibility (inside) (21) confirmed that >90% of these IOVs were properly oriented and resealed.

**PL Scramblase Activity in IOVs—** IOVs were suspended in 100 mM Tris, 140 mM KCl, 7.5 mM NaCl, 0.1 mM EDTA, pH 7.4 at 100 μg protein/ml. NBD-PLs (PC or PS) were added to a final 0.25 mol % of total PL. After incorporation of label, IOVs were incubated in the presence of 0–2 mM CaCl\(_2\) for 15 min at 37°C. For each sample, percentage of NBD-PL distributed from the external to inward-facing membrane leaflet was determined by the bovine serum albumin "back-exchange" method as described previously (16). Scramblase activity was evaluated by comparing the amount of NBD-PL moved to the inward-facing leaflet of IOVs in the presence of Ca\(^{2+}\) to that observed in the presence of 1 mM EGTA. Due to the known transport of dithionite by the erythrocyte membrane anion exchanger (22), the transmembrane distribution of NBD-PL in IOVs was measured using the method of the exchange of external NBD-PL in 1% albumin back-exchange samples. Compared, these two methods for monitoring transmembrane distribution of NBD-PL (dithionite quenching and albumin back-extraction) have been shown to yield equivalent results (11).
RESULTS AND DISCUSSION

Assay for Scramblase Activity in Reconstituted Liposomes—
\( \text{Ca}^{2+} \)-dependent phospholipid scramblase activity has been shown to persist in resealed erythrocyte ghosts and in inside-out membrane vesicles derived from erythrocyte ghosts, suggesting that this activity results from direct interaction of \( \text{Ca}^{2+} \) with an integral membrane component. In order to undertake reconstitution of this activity from detergent extracts of erythrocyte membranes and to monitor enrichment of activity through subsequent steps of purification, we utilized a fluorescence assay to monitor transbilayer movement of NBD-labeled phospholipids in proteoliposomes reconstituted from exogenous phospholipids and erythrocyte membrane proteins. In this assay, the distribution of NBD-labeled phospholipid (PC or PS) between inner and outer membrane leaflet of the vesicle is determined by selective quenching of fluorescence arising from NBD located in the outer leaflet by the membrane-impermeant quencher dithionite (16, 20). In vesicles reconstituted with NBD-PS exclusively in the outer leaflet, addition of dithionite resulted in virtually complete (>98%) quenching of vesicle fluorescence. By contrast, in vesicles prepared with NBD-PS randomly distributed throughout the membrane, quenching of fluorescence arising from NBD-PS in the inner leaflet of NBD-PS-P in the inner leaflet of inside-labeled vesicles appeared accessible to dithionite quenching, confirming that virtually all vesicles were impermeable to the quencher. Similar results were obtained with NBD-PC (not shown).

Purification and Membrane Reconstitution of Erythrocyte PL Scramblase—Human erythrocyte membrane proteins depleted of cytoskeleton were solubilized with OG and initially fractionated by anion exchange chromatography (Fig. 1). Each eluting fraction was reconstituted into PL vesicles and then 0.25 mol % NBD-PS added to the outer leaflet. After incubation in the presence of either 0 or 2 mM \( \text{Ca}^{2+} \), the distribution of NBD-PS between inner and outer leaflets was determined (see “Experimental Procedures” and below). A \( \text{Ca}^{2+} \)-dependent movement of NBD-PS from outer to inner membrane leaflets detected by a difference in fluorescence quenching by dithionite for proteoliposomes preincubated in \( \text{Ca}^{2+} \) versus EGTA was observed in only those vesicles reconstituted with proteins eluting from Poros 20 HQ between 110 mM and 200 mM NaCl. Such activity was not detected in any other column fraction, including those enriched with the predominant erythrocyte membrane band 3 protein. Purification of NBD-PS in proteoliposomes preincubated in \( \text{Ca}^{2+} \) versus EGTA was observed in only those vesicles reconstituted with proteins eluting from Poros 20 HQ between 110 mM and 200 mM NaCl. Such activity was not detected in any other column fraction, including those enriched with the predominant erythrocyte membrane band 3 protein, nor was this activity found in unbound flow-through fractions (not shown). Of note, erythrocyte band 3 protein has been reported to exhibit intrinsic “flipping activity” for anionic phospholipids, based on the capacity of the anion transporter inhibitor 4,4’-disothiocyanostilbene-2,2’-disulfonic acid to further attenuate the slow spontaneous \( \text{Ca}^{2+} \)-independent migration of PL across the erythrocyte membrane (23).

After negative absorption against Poros HS cation exchange resin followed by chromatography on reactive red 120 (see “Experimental Procedures”), the eluting PL scramblase-containing fractions were pooled, concentrated, and gel-filtered on Bio-Sil SEC 250. As is evident from Fig. 2, PL scramblase activity was found to co-purify with a ~37-kDa protein band that we were able to enrich to apparent homogeneity. This PL scramblase lost >80% of its activity upon incubation for 3 h at 37 °C in the presence of trypsin, while activity was preserved if soybean trypsin inhibitor was additionally present, confirming that activity detected in the reconstituted proteoliposome derived from the purified protein fraction and not other potentially co-eluting material (not shown). In this context, PL scramblase activity has recently been attributed to an interaction of \( \text{Ca}^{2+} \) directly with membrane lipid. As noted above, Sulpice et al. (19) concluded that \( \text{Ca}^{2+} \) binding to \( \text{PIP}_{2} \) located in the inner leaflet of the erythrocyte membrane induces transbilayer PL scrambling, although this conclusion was not supported by a subsequent study (16). In the present study, no \( \text{Ca}^{2+} \)-induced PL scrambling was observed in PC/PS liposomes containing up to 4 mol % \( \text{PIP}_{2} \), nor did incorporation of 4 mol % \( \text{PIP}_{2} \) alter the observed \( \text{Ca}^{2+} \)-induced transmembrane movement of NBD-PL in proteoliposomes containing purified PL scramblase (not shown). Alternatively, Bratton et al. concluded that cytoplasmic polyamines contribute to maintenance of PL asymmetry by shielding anionic head groups from \( \text{Ca}^{2+} \) and that enhanced transbilayer movement of PL might arise through loss of polyamine-membrane associations at increased intracellular \( \text{Ca}^{2+} \) (12). Although our studies do not address a potential influence of polyamines on PL scramblase activity, it should be noted that cytotoxic polyamines would be removed through our lysis and solubilization procedures. Finally, sensitivity of PL scramblase to inactivation by trypsin excludes the possibility that the activity we observe arises from contaminating \( \text{PIP}_{2} \).
or polyamine. Although data of Fig. 2 suggest that PL scramblase is a low abundance membrane polypeptide of \( \approx 37 \) kDa, we cannot now exclude the possibility that the observed activity resides in a trace co-purifying membrane protein that goes undetected by silver staining.

Characteristics of PL Transport Mediated by Isolated PL Scramblase. Proteoliposomes reconstituted with purified PL scramblase remained impermeant to dithionite and stable to spontaneous NBD-PS exchange in the absence of Ca\(^{2+}\) (Fig. 3). As also illustrated by Fig. 3, Ca\(^{2+}\)-induced PL scramblase activity was observed to be bidirectional, induced sequestration of NBD-PS initially distributed only in the outer membrane leaflet equaling induced exposure of NBD-PS initially distributed only in the inner leaflet. In proteoliposomes prepared with NBD-PS randomly distributed between membrane leaflets, no movement of probe was detected upon Ca\(^{2+}\) addition, consistent with bidirectional PL exchange between membrane leaflets. Similar results were obtained when NBD-PC substituted for NBD-PS, and movement of either NBD-labeled PL was unaffected by replacement of matrix PS by phosphatidic acid (data not shown). Experiments similar to those described in Fig. 3 were performed with proteoliposomes that had been resealed in the presence of 2 mM Ca\(^{2+}\). The addition of Ca\(^{2+}\) inside the proteoliposomes increased both the inward movement (observed with outside-labeled proteoliposomes) and outward movement (observed with inside-labeled proteoliposomes) of NBD-PL by a factor of 1.2-1.5-fold above that observed in the presence of 2 mM external Ca\(^{2+}\) alone, suggesting that additional scramblase molecules were activated under these conditions. As was also noted above, no movement of probe was detected in symmetrically labeled proteoliposomes resealed to Ca\(^{2+}\) (not shown). These data suggest that in the reconstituted proteoliposomes as much as one-third of the membrane-incorporated protein is oriented to expose Ca\(^{2+}\) binding site(s) only on the internal face of the lipid bilayer and cannot be activated by external Ca\(^{2+}\).

Taken together, these results imply that the PL scramblase activity exhibited by lipid vesicles reconstituted with this 37-kDa protein is nonselective for PS versus PC and facilitates both inward and outward movement of PL between membrane leaflets. Although it has been suggested that elevation of cytosolic Ca\(^{2+}\) initiates a vectorial movement of PS and PE from inner to outer plasma membrane leaflets (7, 13), more recent data suggest that this apparent egress of inner leaflet PS arises through random bidirectional exchange of PL residing in both leaflets of the plasma membrane (4, 6, 11). The fact that these proteoliposomes remain impermeant to dithionite (Fig. 3) excludes the possibility that this apparently random and bidirectional movement of lipid arises through lytic disruption of lipid bilayer. Nevertheless, we cannot rule out the possibility that an initially vectorial transport of PL in these small vesicles is masked by a counterbalancing exchange to maintain mass balance between membrane leaflets, as might be imposed by head group packing constraints.

The mechanism by which Ca\(^{2+}\) interaction with this protein initiates transbilayer movement of PL remains unresolved. To be noted, we observed only a requirement for Ca\(^{2+}\) with no requirement for either Mg\(^{2+}\) or ATP. In presence of 2 mM Ca\(^{2+}\), lipid movement catalyzed by PL scramblase obeyed pseudo-first order kinetics with initial rates that increased with protein concentration, consistent with a facilitated transport or carrier mechanism (Fig. 4). Derived rate constants for transbilayer lipid movement (at 37 °C) ranged from 0.15 to 0.74 h\(^{-1}\) over an 8-fold range of protein/lipid ratio. Whereas this activity deviated from anticipated linearity with increased added protein per lipid (Fig. 4, inset), this may reflect protein aggregation at high concentration or an inherent limitation in
efficiency of protein reconstitution into the liposome membrane (see below). The data of Fig. 4 also indicate that the amount of NBD-PL scrambled at t = ∞ increases with protein/lipid ratio and never achieves 50%, the maximal fluorescence quenching expected if all NBD-PL molecules in all vesicles were completely equilibrated between the two membrane leaflets. These results in part reflect the subset of protein reconstituted with inward-facing Ca$^{2+}$-binding site(s) (see above). These data also suggest that even at the highest protein concentrations achieved some liposomes are formed without any functional scramblase activity, and the NBD-PL in these scramblase-free vesicles remains asymmetrically distributed under the conditions of our assay.

To exclude the possibility that the observed kinetics reflected a time-dependent inactivation of the protein during the 3-h incubation at 37 °C, we compared the kinetics of NBD-PL movement when probe was added to the vesicles after an initial 4-h preincubation at 37 °C in the presence of 2 mM Ca$^{2+}$. Addition of NBD-PS (after 4 h of incubation of proteoliposomes in 2 mM Ca$^{2+}$) resulted in transmembrane movement of NBD-PS identical with that observed under conditions shown in Fig. 4 (addition of Ca$^{2+}$ at t = 0), indicating that there was no detectable loss of scramblase activity under these conditions of preincubation (data not shown). The finding that the reconstituted NBD-PL scramblase remains fully active after prolonged preincubation of the proteoliposomes with Ca$^{2+}$ also excludes the possibility that the observed transmembrane redistribution of NBD-PL we detect upon addition of Ca$^{2+}$ to NBD-PL-labeled proteoliposomes (e.g. Fig. 4) reflects redistribution of phospholipid secondary to Ca$^{2+}$-induced fusion of the vesicles. The apparent rate of Ca$^{2+}$-evoked transbilayer movement attained in the reconstituted system (t$_{1/2}$ ~ 2 h; Fig. 4) is approximately one-fourth of the observed rate of PL scrambling (t$_{1/2}$ ~ 0.5 h; see Ref. 4) induced in Ca$^{2+}$-ionophore treated erythrocytes, suggesting somewhat reduced activity in the reconstituted system. Whether this reduced activity reflects partial denaturation of PL scramblase or our inability to incorporate amounts of this protein equivalent to that present in the erythrocyte membrane remains to be determined.

**Calcium Dependence**—In order to confirm that the activity of reconstituted PL scramblase mimicked intrinsic PL scramblase activity of the erythrocyte membrane, we compared Ca$^{2+}$ dependence of PL movement in proteoliposomes reconstituted with purified protein to that observed in erythrocyte IOVs (Fig. 5). In both IOVs and isolated protein-reconstituted liposomes, PL scramblase activity was found to obey a sigmoidal dose response to Ca$^{2+}$, with saturation attained at ~100 μM [Ca$^{2+}$] and half-maximal activities between 20 and 60 μM Ca$^{2+}$. In both cases, little effect of Mg$^{2+}$ was observed. These results are similar to data previously reported for Ca$^{2+}$-induced movement of plasma membrane PL in ionophore-treated erythrocytes, resealed erythrocyte ghosts, and other cells (24, 25).

PL scramblase activity found in erythrocyte membranes is also observed in platelets, lymphocytes, and other cells and is thought to play a central role in initiation of fibrin clot formation and in recognition of apoptotic and injured cells by the reticuloendothelial system. It is not known whether the same protein is responsible for PL scramblase activity found in other cells or whether such proteins are cell-specific. A protein common to all blood cells is suggested by the observation that the genetic defect in PL scramblase activity arising in Scott syndrome affects all hematologic lineages (16, 24). Further insight into the cellular distribution of the 37-kDa protein that we have now isolated from normal erythrocytes awaits purification of sufficient protein for sequencing and development of specific antibodies.

Acknowledgment—We thank Robert A. Luhm for technical assistance with protein purification.

**REFERENCES**

1. Schrott, A. J., and Zwaal, R. F. A. (1991) Biochim. Biophys. Acta 1071, 313–329
2. Devaux, P. (1991) Biochemistry 30, 1163–1173
3. Connor, J., and Schrott, A. J. (1987) Biochemistry 26, 5099–5105
4. Williamson, P., Kulick, A., Zachowski, A., Schlegel, R. A., and Devaux, P. F. (1992) Biochemistry 31, 6355–6360
5. Chang, C.-P., Zhao, J., Wiedmer, T., and Sims, P. J., (1993) J. Biol. Chem. 268, 7171–7178
6. Smeets, E. F., Comfurius, P., Bevers, E. M., and Zwaal, R. F. A. (1994) Biochemistry 33, 281–286
7. Basset, F., Gaffet, P., Rendu, F., and Bienvenu, A. (1993) Biochemistry 32, 2337–2344
8. Bevers, E. M., Comfurius, P., and Zwaal, R. F. A. (1992) Blood Rev. 5, 146–154
9. Sims, P. J., Falconi, E., Wiedmer, T., and Shattil, S. J. (1988) J. Biol. Chem. 263, 18205–18212
10. Fadak, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) J. Immunol. 148, 2207–2216
11. Williamson, P., Bevers, E. M., Smeets, E. F., Comfurius, P., Schlegel, R. A., and
A Membrane Protein with Phospholipid Scramblase Activity

12. Bratton, D. L. (1994) J. Biol. Chem. 269, 22517–22523
13. Gaffet, P., Bettache, N., and Bienvenue, A. (1995) Biochemistry 34, 6762–6769
14. Comfurius, P., Senden, J. M., Tilly, R. H., Schroit, A. J., Bevers, E. M., and Zwaal, R. F. (1990) Biochim. Biophys. Acta 1026, 153–160
15. Martin, D. W., and Jesty, J. (1995) J. Biol. Chem. 270, 10468–10474
16. Bevers, E. M., Wiedmer, T., Comfurius, P., Zhao, J., Smeets, E. F., Schlegel, R. A., Schroit, A. J., Weiss, H. J., Williamson, P., Zwaal, R. F. A., and Sims, P. J. (1995) Blood 86, 1983–1991
17. Fox, J. E. B., Reynolds, C. C., and Austin, C. D. (1990) Blood 76, 2510–2519
18. Wiedmer, T., Shattil, S. J., Cunningham, M., and Sims, P. J. (1990) Biochemistry 29, 623–632
19. Sulpice, J. C., Zachowski, A., Devaux, P. F., and Giraud, F. (1994) J. Biol. Chem. 269, 6347–6354
20. McIntyre, J. C., and Sleight, R. G. (1991) Biochemistry 30, 11819–11827
21. Steck, T. L., and Kant, J. A. (1974) Methods Enzymol. 31, 172–180
22. Pomorski, T., Herrmann, A., Zachowski, A., Devaux, P. F., and Muller, P. (1994) Mol. Membr. Biol. 11, 39–44
23. Vondenhof, A., Osliender, A., Deuticke, B., and Haest, C. W. (1994) Biochemistry 33, 4517–4520
24. Kojima, H., Newton-Nash, D., Weiss, H. J., Sims, P. J., and Wiedmer, T. (1994) J. Clin. Invest. 94, 2237–2243
25. Verhoven, B., Schlegel, R. A., and Williamson, P. (1992) Biochim. Biophys. Acta 1104, 15–23