Effect of Nonbilayer Lipids on Membrane Binding and Insertion of the Catalytic Domain of Leader Peptidase†

Els van den Brink-van der Laan,*,‡ Ross E. Dalbey,§ Rudy A. Demel,‡ J. Antoinette Killian,‡ and Ben de Kruijff‡

Department Biochemistry of Membranes, Center for Biomembranes and Lipid Enzymology, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH, Utrecht, The Netherlands, and Department of Chemistry, Ohio State University, Columbus, Ohio 43210

Received December 21, 2000; Revised Manuscript Received May 24, 2001

ABSTRACT: Biological membranes contain a substantial amount of "nonbilayer lipids", which have a tendency to form nonlamellar phases. In this study the hypothesis was tested that the presence of nonbilayer lipids in a membrane, due to their overall small headgroup, results in a lower packing density in the headgroup region, which might facilitate the interfacial insertion of proteins. Using the catalytic domain of leader peptidase (Δ2−75) from *Escherichia coli* as a model protein, we studied the lipid class dependence of its insertion and binding. In both lipid monolayers and vesicles, the membrane binding of (catalytically active) Δ2−75 was much higher for the nonbilayer lipid DOPE compared to the bilayer lipid DOPC. For the nonbilayer lipids DOG and MGDG a similar effect was observed as for DOPE, strongly suggesting that no specific interactions are involved but that the small headgroups create hydrophobic interfacial insertion sites. On the basis of the results of the monolayer experiments, calculations were performed to estimate the space between the lipid headgroups accessible to the protein. We estimate a maximal size of the insertion sites of 15 ± 7 Å^2/lipid molecule for DOPE, relative to DOPC. The size of the insertion sites decreases with an increase in headgroup size. These results show that nonbilayer lipids stimulate the membrane insertion of Δ2−75 and support the idea that such lipids create insertion sites by reducing the packing density at the membrane−water interface. It is suggested that PE in the bacterial membrane facilitates membrane insertion of the catalytic domain of leader peptidase, allowing the protein to reach the cleavage site in preproteins.

Although biological membranes maintain an overall bilayer structure under physiological conditions, they contain substantial amount of lipids that have a strong preference to organize in nonlamellar structures, such as the inverted hexagonal phase. These "nonbilayer lipids" have a small headgroup area compared to the area of the acyl chains, resulting in an effective conical shape, in contrast to bilayer lipids, which have an effective cylindrical shape (*1, 2*). It is known for the microorganisms *Acholeplasma laidlawii* (*3*), *Clostridium butyricum* (*4*), and *Escherichia coli* (*5*) that they adapt the relative amounts of bilayer and nonbilayer lipids in their membranes in response to the environmental conditions, to maintain the physical state of the lipids always close to a lamellar−nonlamellar phase transition. Moreover, this tight regulation of lipid composition is essential for growth: the *E. coli* strain AD93, which is unable to synthesize the nonbilayer lipid phosphatidylethanolamine (PE),[1] is only able to grow in the presence of specific divalent cations, under which conditions cardiolipin, in combination with these cations, can take over the role of PE as nonbilayer lipid (*6, 7*).

Despite their importance for the cell, very little is known about the molecular functioning of these nonbilayer lipids. Two possibilities can be considered. First, the physical state of the membrane lipids close to the bilayer−nonbilayer transition allows the membrane to readily undergo local rearrangements needed for function, for instance, membrane fusion. The other possibility is that the presence of nonbilayer lipids gives rise to special packing properties of the membrane, which are required for proper functioning of proteins.

Indeed, for an increasing number of membrane proteins it is known that nonbilayer lipids are important for their function. This is the case not only for integral membrane proteins, such as alamethicin (*8*) and the bacterial protein translocase (*9, 10*), but also for peripheral membrane proteins such as diglucosyldiacylglycerol synthase (*3, 11*), protein

† This work was supported by the Dutch Foundation for Fundamental Research on Matter (FOM).

* Corresponding author: fax (31)(30)2522478; tel (31)(30)2533553; e-mail e.vandenbrink@chem.uu.nl.

‡ Utrecht University.

§ Ohio State University.

[1] Abbreviations: BSA, bovine serum albumin; DGDG, digalactosyldiacylglycerol; DOG, 1,2-dioleoylglycerol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPENMe, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methyl; DOPENMe$_2$, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*,*N*-dimethyl; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactoside; Δ2−75, catalytic domain of leader peptidase; LUVs, large unilamellar vesicles prepared by extrusion techniques; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi002903a CCC: $20.00
Published on Web 07/19/2001

kinase C (*12*), and CTP:phosphocholine cytidyltransferase (*13*). How can the presence of nonbilayer lipids affect the function of membrane proteins? Cone-shaped nonbilayer lipids have the tendency to organize into structures with a negative curvature. When these lipids are constrained in a flat bilayer, because the opposite monolayer has the same intrinsic negative curvature, this results in bilayer frustration *(14*). These bilayers will have an increased packing density at the center of the membrane and a lower packing density at the membrane interface, as compared to bilayer lipids. It is possible that such a packing density profile is essential for membrane proteins to function. One aspect of this hypothesis would be that the lower packing density at the membrane−water interface by the presence of nonbilayer lipids might facilitate the interfacial insertion of proteins.

First insight into this possibility was obtained by Van Klompenburg et al. (*15*) in a study on leader peptidase from *Escherichia coli*. Leader peptidase is an integral membrane protein, consisting of two transmembrane helices and a large periplasmic region, which contains the active site. It plays a role in the protein translocation pathway, where it cleaves the signal peptide from translocated precursor proteins. Van Klompenburg (*15*) used a catalytically active variant of leader peptidase (Δ2−75) in which the transmembrane helices were removed. It was shown that despite the absence of these helices this protein is able to penetrate into lipid monolayers due to the presence of a hydrophobic segment (residues 83−98), with a preference for the nonbilayer lipid dioleoylphosphatidylethanolamine (DOPE), compared to dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) (both bilayer-preferring lipids). The subsequent elucidation of the crystal structure of this fragment of leader peptidase visualized this hydrophobic segment as a large exposed hydrophobic surface including the substrate binding site and catalytic center (serine 90), which was suggested to be the membrane association surface (*16*). These properties make Δ2−75 a good model protein to study the effect of nonbilayer lipids on the membrane binding and function of peripheral proteins on a molecular level.

Although the data of Van Klompenburg et al. (*15*) clearly show an interaction between Δ2−75 and DOPE, the question is still open whether this is a specific interaction with DOPE or intrinsic to the nonbilayer nature of this lipid. Possible specific effects of PE, which have been reported in the literature, are the formation of hydrogen bonds (*17*), the induction of domains enriched in negatively charged phospholipids in mixed membranes of PE with negatively charged phospholipids (*18*), and a chaperone-like function of PE in the folding of membrane proteins (*19*). To determine whether the interaction of Δ2−75 with DOPE is specific for this lipid or a general property of nonbilayer lipids, we studied the interaction between Δ2−75 and different bilayer and nonbilayer lipids, using monolayer techniques as well as vesicle binding experiments.

We found that the protein binds to membranes with a preference for lipids with a small lipid headgroup, independent of the exact chemical structure of the headgroup. Moreover, on the basis of monolayer experiments we could estimate for the first time the size of the protein insertion sites between the phospholipid headgroups, relative to DOPC. We found an increase of the size of the insertion sites in the presence of nonbilayer lipids, with a maximum value of 15 ± 7 Å^2/lipid molecule for a monolayer of pure DOPE (relative to DOPC).

EXPERIMENTAL PROCEDURES

Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methyl (DOPENMe), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*,*N*-dimethyl (DOPENMe$_2$), and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol (DOPG) were purchased from Avanti Polar Lipids Inc. Plant digalactosyldiacylglycerol (DGDG) and plant monogalactosyldiacylglycerol (MGDG) were obtained from Larodan Fine Chemicals AB. 1,2-Dioleoylglycerol (DOG) was purchased from Doosan Serdary Research Laboratories, *n*-octyl β-D-glucopyranoside (octyl glucoside) and isopropyl thiogalactoside (IPTG) were obtained from Sigma, and ^{14}C formaldehyde was from Amersham Pharmacia Biotech (specific activity 55 mCi/mmol). All other chemicals were of the highest purity commercially available. PrePhoE was purified as described before (*20*).

Purification of Δ2−75. For the purification of Δ2−75 a purification procedure was followed that was mainly based on the protocol of Kuo et al. (*21*), with some small modifications. The main modification is that detergent is left out, as was already done by Van Klompenburg et al. (*15*). Some other modifications are based on the slightly different protocol of Paetzel et al. (*22*). For clarity, the complete purification procedure is given below.

The protein was overexpressed in *E. coli* BL21(DE3) containing the gene encoding Δ2−75 subcloned into the pET-3d vector. Cells were grown in LB medium supplemented with 100 μg/mL ampicillin at 37 °C. Induction with 0.5 mM IPTG at an OD_{600} of 0.8 resulted in accumulation of Δ2−75 in inclusion bodies within the cytoplasm of the cells. The cell growth was continued for 4 h at 37 °C. The cells were pelleted by centrifugation, resuspended in 20 mM Tris-HCl, pH 7.4, and disrupted by passing through a French press at 10 000−20 000 psi. The cell lysate was centrifuged at 12000*g* and the pellet was washed at least four times with 20 mM Tris-HCl, pH 7.4. The inclusion bodies were solubilized in 6 M guanidine hydrochloride and 20 mM Tris-HCl, pH 7.4, at room temperature with stirring for 15 min. This solution was applied to an S-100 column equilibrated with 6 M guanidine hydrochloride and 20 mM Tris-HCl, pH 7.4. The fractions containing Δ2−75 were diluted 3-fold into 20 mM Tris-HCl, pH 7.4, and dialyzed three times overnight against 20 mM Tris-HCl, pH 7.4, at 4 °C. After dialysis, the protein solution was centrifuged at 15000*g* for 1 h to remove any protein aggregates. The refolded protein was concentrated with Amicon Centricon-10 concentrators. The protein solutions, typically in a concentration of 0.1−0.2 mg/mL, were stored at −20 °C. The purity of the protein was estimated to be greater than 95% from a Coomassie blue-stained SDS−polyacrylamide gel. Before any experiment, the protein solution was thawed and ultracentrifuged for 30 min at 236000*g* at 4 °C to remove protein aggregates. The activity of Δ2−75 was measured in a processing assay of the precursor protein prePhoE in an octyl glucoside/DOPG system as described (*23*) except that nonradioactive prePhoE was used as substrate and the samples were analyzed by SDS−PAGE followed by western blotting with PhoE-

antibodies. We measured a processing efficiency of 30% after 1 h compared to 65% for wild-type leader peptidase. This is in agreement with results of Tschantz et al. (*24*), who found that the activity of this variant is somewhat lower than the activity of wild-type leader peptidase.

^{14}C Labeling of Δ2−75. ^{14}C Labeling of Δ2−75 was performed by reductive methylation of the lysine residues, based on the procedure of Dottavio-Martin and Ravel (*25*). This labeling method does not affect the charge of the lysine side-chains. Δ2−75 in 20 mM NaH_2PO_4–Na_2HPO_4, pH 7.4, was incubated with 20 mM $NaCNBH_3$ and ^{14}C formaldehyde (56 Ci/mol, 5:1 molar ratio with respect to protein) for 1 h at room temperature, with constant slow shaking. Because of the formation of protein aggregates during the labeling reaction, the labeled protein was unfolded and refolded again after the labeling. For the unfolding of the protein, guanidine hydrochloride was added at a final concentration of 6 M. Subsequently, the protein was refolded by a 3-fold dilution of the protein solution into 20 mM Tris-HCl, pH 7.4, and dialyzed three times overnight against 20 mM Tris-HCl, pH 7.4, at 4 °C. After the protein solution was concentrated by embedding the dialysis bag in dry Sephadex G-200 at 4 °C, the protein solution was centrifuged at 15000*g* for 1 h. The resulting Δ2−75 preparation had a specific radioactivity of 5060 dpm/μg, which corresponded to an average of 1.2 methylated amino groups/protein. The processing efficiency of the labeled protein, measured as described above, was 23% after 1 h compared to 30% for the unlabeled protein.

Monolayer Experiments. Protein-induced surface pressure changes of a monolayer of phospholipids at constant area were measured by the (platinum) Wilhelmy plate method (*26*) at room temperature with a Cahn D202 microbalance. The subphase was continuously stirred with a magnetic bar, placed in a well in the bottom of a Teflon dish. This Teflon dish, with a volume of 5 mL and a surface area of 8.81 cm^2, was filled with 5 mM EDTA and 20 mM Tris-HCl, pH 7.4. The monomolecular lipid layers were spread from a chloroform solution at the air/buffer interface to give an initial surface pressure of 25 mN/m. A saturating amount of ^{14}C-Δ2−75 (4 μg/mL) was added through a small injection hole. The surface pressure was measured during 1 h. Subsequently, the subphase was washed with 50 mL of 5 mM EDTA and 20 mM Tris-HCl, pH 7.4, to remove unbound protein molecules. The surface pressure did not change during washing, indicating that the monolayer-bound protein was not removed. Monolayers were collected by aspiration through a glass capillary directly into a scintillation vial, while the molecular area was manually decreased with a movable barrier, with an efficiency of 90% (*27*). The amount of monolayer-bound protein was determined by liquid scintillation counting of the collected monolayer; the results were corrected for the radioactivity measured in an equal volume of subphase.

Vesicle Binding Assay. Dry lipid films were hydrated in 50 mM NaCl and 20 mM Tris-HCl, pH 7.4. The dispersions were frozen and thawed 10 times and extruded through two stacked 400 nm pore size polycarbonate filters (Millipore) as described *(28)* to form large unilamellar vesicles (LUVs). These vesicles (0.25−4 mM, on a phosphorus basis) were incubated for 1 h at room temperature with a mixture of ^{14}C-Δ2−75 and unlabeled Δ2−75 in a total protein concentration of 50 μg/mL. Vesicles were pelleted by ultracentrifugation for 30 min at 236000*g* at 4 °C in a TLA 100.2 rotor in a TL100 ultracentrifuge (Beckmann Instruments Inc., Palo Alto, CA). Under these conditions, less than 5% of the phospholipids remained in the supernatant, as was determined by a phosphorus assay (*29*). Eighty percent of the supernatant was removed, while the pellet was resuspended in the remaining 20% supernatant. The amount of protein in the pellet and supernatant fraction was determined by liquid scintillation counting. The amount of protein was calculated from the protein concentration in the pellet fraction, after subtraction of the protein content of the 20% supernatant that was still present in the pellet fraction. The amount of bound protein was also corrected for the presence of protein aggregates in the pellet, by subtracting the amount of protein in the pellet of a sample without vesicles, which was typically 5−10% of the total amount of protein.

Activity of Membrane-Bound and Unbound Δ2−75. A vesicle binding assay was performed as described above, with 50 μg/mL Δ2−75 and 2 mM lipid vesicles (25% DOPG, 75% DOPE). After incubation at room temperature and centrifugation, the protein concentration was determined for the pellet and supernatant fractions. The pellet fraction was then diluted to the same protein concentration as the supernatant fraction (24 μg/mL), while lipids (5 mM) were added to the supernatant fraction to obtain the same lipid concentration as the pellet fraction (5 mM). Finally 50 mM octyl glucoside was added to both fractions to form mixed micelles. The activity of Δ2−75 in both fractions was measured in a processing assay of prePhoE as described above. Samples of 200 μL of both fractions were incubated with 2 μL of prePhoE (1.7 mg/mL in octyl glucoside) for 2 h at room temperature. After acetone precipitation in the presence of bovine serum albumin (BSA), the samples were analyzed by SDS−PAGE, followed by western blotting with PhoE-antibodies. The intensities of the bands were quantified by densitometry (GS-700 imaging densitometer, Bio-Rad). The processing efficiency was calculated as the ratio between the intensity of the PhoE band and the sum of the PhoE and prePhoE bands.

RESULTS

Monolayer Binding and Insertion of Δ2−75. In this study we used the catalytic domain of leader peptidase (Δ2−75) as a model protein to investigate the effect of nonbilayer lipids on membrane binding and insertion of proteins. The purification of Δ2−75 was carried out essentially as described before without detergent, but with some minor modifications as specified under Experimental Procedures. First, we verified that this protein discriminates between dioleoylphosphatidylethanolamine (DOPE) and dioleoylphosphatidylcholine (DOPC) in a monolayer experiment. A monolayer of either DOPC or DOPE was spread at a surface pressure of 25 mN/m, after which a saturating amount of protein was injected into the subphase, while the surface pressure was measured continuously. Figure 1 shows that the surface pressure increased as a result of penetration of protein in the monolayer. The surface pressure increase leveled off within 60 min of incubation. For DOPE a final surface pressure increase of 9 mN/m was reached, while this increase was only 4 mN/m for DOPC. These values are in good agreement with the results of Van Klompenburg et al. (*15*) and demonstrate that the modifications in the purification

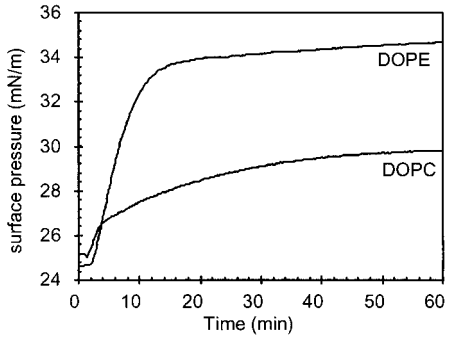

FIGURE 1: Surface pressure increase induced by interaction of Δ2−75 with lipid monolayers, composed of DOPC or DOPE. Monolayers were spread at an initial surface pressure of 25 mN/m. A saturating amount of protein (4 μg/mL) was injected into the subphase after 2 min.

procedure do not affect the preferential insertion of the protein into the DOPE monolayer.

Similar experiments were done for different bilayer and nonbilayer lipids. First, the *N*-methylated derivatives of DOPE, dioleoylphosphatidylethanolamine-*N*-methyl (DOPENMe) and dioleoylphosphatidylethanolamine-*N*,*N*-dimethyl ($DOPENMe_2$), were tested to determine the specificity of the protein−monolayer interaction. These lipids all have similar charged headgroups but are different in size. The final surface pressure increases after 60 min of incubation are shown in Figure 2 (black bars). No significant difference was observed between the surface pressure increase for DOPC, $DOPENMe_2$, and DOPENMe, whereas the surface pressure increase for DOPE was significantly higher. To further test the specificity of the interaction of Δ2−75 with monolayers, other chemically unrelated lipids were tested, with variation in lipid headgroup size. The results are also shown in Figure 2 (white bars). These results, both for the *N*-methylated derivatives of PE and the other (non)bilayer lipids, show the general trend that the surface pressure increase is higher in the case of a smaller lipid headgroup. Digalactosyldiacylglycerol (DGDG), like DOPC, is a bilayer lipid but has a large uncharged carbohydrate headgroup. Δ2−75 interacted with similar efficiency with monolayers of this lipid as with monolayers of DOPC. However, in the case of monogalactosyldiacylglycerol (MGDG), which contains one galactose unit less than DGDG, the surface pressure increase was greatly enhanced. Complete removal of the headgroup as in dioleoylglycerol (DOG) resulted in an even higher surface pressure increase.

When examining these suface pressure increases, it should be noted, however, that the effect of a protein on the surface pressure is determined both by the ability of a protein to insert between the lipid molecules of the monolayer and cause a surface pressure increase and by the amount of protein molecules associated with the monolayer. To determine the amount of monolayer-bound protein, radioactively labeled protein was used. The protein/lipid ratio (*P*/*L*) is given by

$$P/L = \frac{N_p}{N_1} = \frac{N_p A_{1,\pi_i}}{A_m} \quad (1)$$

where N_p is the total number of protein molecules finally bound to the monolayer, determined by measuring radio-

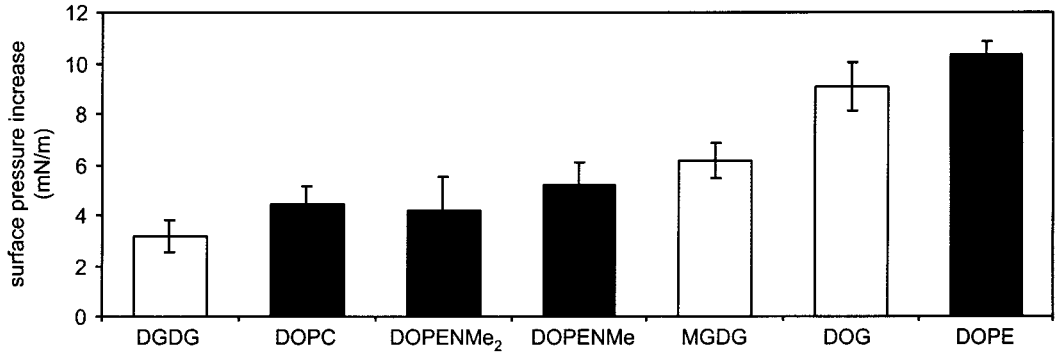

FIGURE 2: Interaction of Δ2−75 with different lipid monolayers, spread at an initial surface pressure of 25 mN/m. The surface pressure increase 60 min after injection of protein, determined as described under Experimental Procedures, is given for PE and its *N*-methylated derivatives (black bars), as well as for the other (non)bilayer lipids DGDG, MGDG, and DOG (white bars). The data shown are the average of at least 4 independent measurements. Error bars represent the standard deviation.

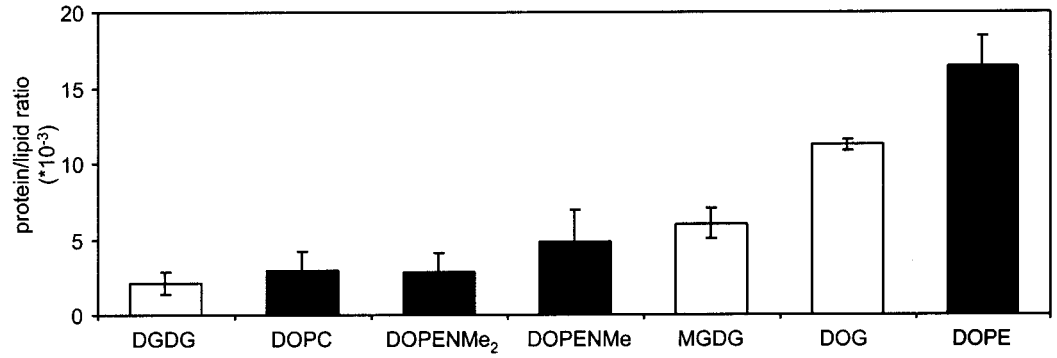

FIGURE 3: Interaction of Δ2−75 with different lipid monolayers, spread at an initial surface pressure of 25 mN/m. The protein/lipid ratio, determined as described under Experimental Procedures, is given for PE and its *N*-methylated derivatives (black bars), as well as for the other (non)bilayer lipids DGDG, MGDG, and DOG (white bars). The data shown are the average of at least 4 independent measurements. Error bars represent the standard deviation.

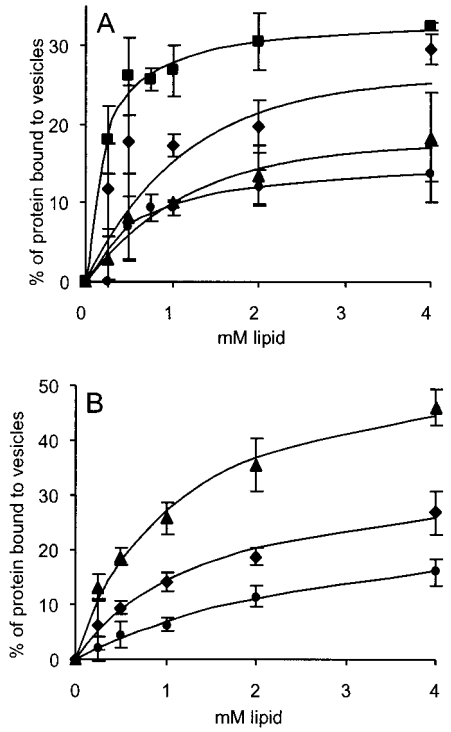

FIGURE 4: Binding of Δ2−75 to LUVs composed of 25% DOPG and 75% zwitterionic or uncharged (phospho)lipids. Binding studies were performed as described under Experimental Procedures. (A) LUVs contained 75% DOPE (■), DOPENMe (◆), DOPENMe$_2$ (▲), or DOPC (●). (B) LUVs were composed of DOPG/DOPC/DOG (5/13/2) (▲), DOPG/DOPC/MGDG (5/11/4) (◆), or DOPG/DOPC/DGDG (5/11/4) (●). The shown data are the average of 4 experiments. Error bars represent the standard deviation. Curves are based on least-squares fits.

activity in the collected monolayer, as described under Experimental Procedures, and N_l is the total number of lipid molecules, as determined from the total area of the monolayer (A_m) and the area per lipid molecule at the initial surface pressure (A_{l,π_i}), based on pressure−area isotherms for each lipid, which were taken from literature (*26*) or measured by ourselves (data not shown). Because of the small dish that is used for the monolayer experiments, this way of calculation of N_l is more accurate than calculation of N_l based on the added amount of lipids. Figure 3 shows that the protein/lipid ratio is maximal for DOPE, while this ratio is lower for the *N*-methylated derivatives of DOPE (black bars). For the other (non)bilayer lipids, DGDG, MGDG, and DOG, a similar trend is observed: Δ2−75 binds more efficiently when the lipid headgroup size is decreased. These results indicate that it is indeed the small headgroup size of a lipid that is the most determining factor for the interaction of Δ2−75 with lipids in monolayers.

Δ2−75 Binding to Vesicles. The strength of the monolayer technique is that it can directly and quantitatively measure the ability of a protein to insert into the lipid layer. However, self-assembled bilayers mimic more closely the lipid organization of biological membranes. Therefore, we also tested the lipid specificity of Δ2−75 binding to large unilamellar vesicles (LUVs) in a centrifugation experiment. Vesicles consisted of 75% zwitterionic or uncharged (non)bilayer lipids with 25% of the negatively charged bilayer lipid DOPG, to allow formation of stable vesicles also in the presence of nonbilayer lipids.

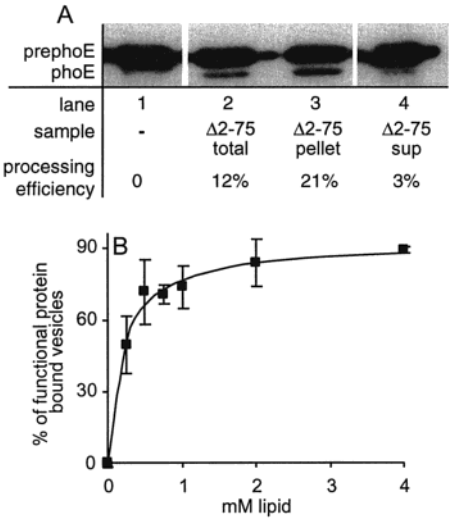

FIGURE 5: (A) Catalytic activity of Δ2−75 in pellet and supernatant fraction, assayed in a processing assay as described under Experimental Procedures. The signal peptide of the precursor protein prePhoE (in mixed micelles) can be cleaved off by Δ2−75, resulting in the protein PhoE with a slightly lower molecular weight, which can be observed as an additional lower band on SDS−PAGE. Lane 1 shows prePhoE incubated without Δ2−75, lane 2 shows the processing of prePhoE by Δ2−75 before centrifugation, and lanes 3 and 4 show the processing of prePhoE by Δ2−75 in the pellet and supernatant fraction, respectively. (B) Binding curve for DOPE/DOPG (3:1) vesicles (also shown in Figure 4A), rescaled for only functional protein (see Results section).

The same lipids were tested as were used for the monolayer experiments. It is shown in Figure 4A for DOPE and its *N*-methylated derivatives that in all cases the amount of membrane-bound Δ2−75 increases for increasing lipid concentration but that the binding saturates around 2 mM lipid. The extent of binding increased with decreasing size of the lipid headgroup. Vesicles containing 75% of the nonbilayer lipid DOPE (small headgroup, ■) bound Δ2−75 up to 32% of the total amount of protein, while this value was only 14% for the bilayer lipid DOPC (large headgroup, ●). In Figure 4B results are shown for the other bilayer and nonbilayer lipids. The nonbilayer lipids DOG and MGDG were incorporated in vesicles at molar fractions of only 10% and 20%, respectively, to avoid instability of the bilayer of the lipid vesicles. For comparison with MGDG, the bilayer lipid DGDG also was incorporated at only 20%. Vesicles containing DGDG (●) showed similar results to vesicles containing DOPC. Both nonbilayer lipids DOG (▲) and MGDG (◆) gave an increased membrane binding of Δ2−75, compared to the bilayer lipids DOPC and DGDG. The membrane binding of Δ2−75 was slightly lower for MGDG compared to DOPE, but higher for DOG. Although only 10% or 20% of these nonbilayer lipids was incorporated in the vesicles, the effect of the presence of these lipids was large. These results show that, not only in the case of lipid monolayers but also for vesicles, the membrane binding of Δ2−75 is enhanced by the presence of nonbilayer lipids. The relative differences between the different lipid species are in good agreement with the monolayer results.

Functionality of Membrane-Bound and Unbound Δ2−75. A remarkable feature of the vesicle binding experiments is that not all protein binds to the vesicles at the highest lipid concentrations that were tested, and it seems that the fitted curves in Figure 4 will saturate at a value lower than 100%,

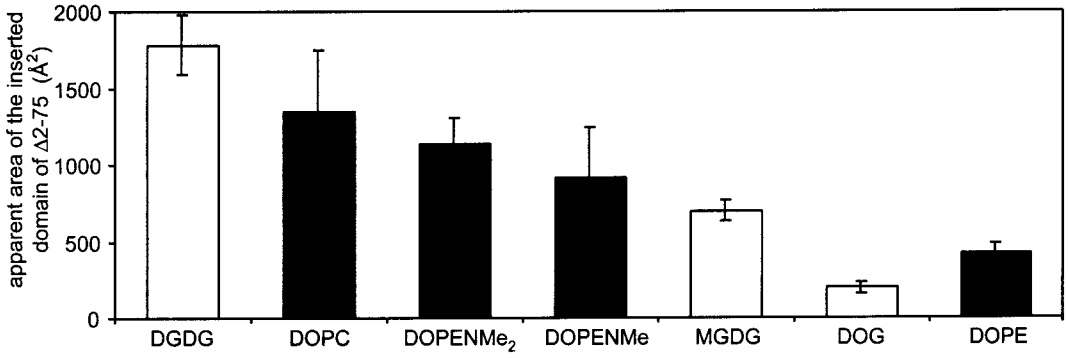

FIGURE 6: Interaction of Δ2−75 with different lipid monolayers, spread at an initial surface pressure of 25 mN/m. The apparent average area of the membrane-inserted domain of Δ2−75 was calculated from the surface pressure increase (Figure 2) and the protein/lipid ratio (Figure 3), as described in the Discussion. This was done for PE and its *N*-methylated derivatives (black bars), as well as for the other (non)bilayer lipids DGDG, MGDG, and DOG (white bars). The data shown are the average of the areas, which were calculated separately for each measurement. Error bars represent the standard deviation.

particularly in the case of DOPE and DOPC. One possible reason for this is heterogeneity in the protein solution, with some proteins that are correctly folded and functional but others that are not. This should be seriously considered given the nature of the protein and the denaturation−renaturation protocol used. To test this possibility we investigated the processing activity of the membrane-bound and unbound protein, assayed in a mixed micellar system as described under Experimental Procedures. We did this for vesicles composed of DOPE/DOPG (3:1), which showed optimal binding of Δ2−75. Figure 5A shows the processing of the precursor protein prePhoE by Δ2−75 after incubation of Δ2−75 with vesicles, before and after subsequent centrifugation, analyzed by SDS−PAGE. The first lane of Figure 5A shows the unprocessed prePhoE. When Δ2−75 cleaves off the signal peptide of prePhoE, this can be observed on SDS−PAGE by a small shift in molecular weight of the protein. The second lane shows this for the total vesicles solution before centrifugation. The pellet fraction (third lane) gave the highest processing efficiency, while that of the supernatant (fourth lane) was very low. The processing efficiencies were quantified (Figure 5A) and the results clearly show that the protein solution is heterogeneous. The activity of the membrane-bound protein is higher than the average activity, suggesting that the affinity of functional protein for membranes is significantly higher than that of nonfunctional protein. The presence of nonfunctional but water-soluble protein is probably the reason for the low amount of membrane-bound protein. When the relative amount of bound protein is corrected for the presence of nonfunctional protein,[2] it appears that almost all functional protein is bound to vesicles at a saturating concentration of vesicles (Figure 5B).

DISCUSSION

The aim of this study was to obtain insight in the effects of nonbilayer lipids on interactions between (peripheral) membrane proteins and membranes. Our hypothesis was that the presence of nonbilayer lipids gives special properties to the membrane, with a lower packing density at the membrane interface, which may facilitate the interfacial insertion of proteins.

We tested this hypothesis using monolayer insertion and vesicle binding experiments with the catalytic domain of leader peptidase as a model protein. The experiments clearly showed that nonbilayer lipids facilitate membrane binding and insertion of the catalytic domain of leader peptidase. An increase in binding and insertion was observed when the headgroup size was decreased going from DOPC to DOPE. All nonbilayer lipids (DOPE, DOG, and MGDG) showed similar effects, which indicate that it is not a result of a specific interaction but that it is the small headgroup of these lipids that is responsible for more protein binding and insertion.

What is the physical process underlying the effect of nonbilayer lipids on membrane binding and insertion of proteins? When these overall conical lipids are mixed with cylindrical bilayer lipids in a bilayer configuration or are spread as a monolayer at the air−water interface, this results in the presence of exposed hydrophobic sites, due to the presence of the small headgroups. These exposed sites are easily filled with membrane-active compounds or proteins, such as Δ2−75. In that case it is to be expected that the hydrophobic patch at Δ2−75 is in contact with the hydrophobic sites in the membrane interface, which is favorable both for the membrane and for the protein.

From a quantitative analysis of the monolayer data, the apparent size of the membrane-inserted domain of the protein ($A_{p,app}$) can be calculated, which is

$$A_{p,app} = \frac{A_{l,\pi_i} - A_{l,\pi_f}}{(P/L)} \quad (2)$$

where A_{l,π_i} and A_{l,π_f} are the area per lipid molecule at the initial and final surface pressure, respectively, based on the

[2] For this correction, a calculation was performed in several steps. First, the activity of the membrane-bound protein was calculated, taking into account the measured processing efficiencies (Figure 5A) and correcting for the presence of protein aggregates (5−10% of the total amount of protein). This results in a processing efficiency of 34% for the membrane-bound Δ2−75. The processing efficiency of the supernatant is only 3% (which is 9% of the value for the membrane-bound protein). From this it can be calculated that if in a volume of 1 mL (protein concentration 50 μg/mL) all 15 μg bound to vesicles is catalytically active and the 2−5 μg of protein that is aggregated is inactive, then of the remaining 30−33 μg in the supernatant only 3 μg is active (9% of the total amount of protein present in the supernatant)], so the total amount of functional protein is 18 μg, and hence the amount of protein bound to the vesicles corresponds to 83% [= (15 μg/18 μg) × 100%] of the total amount of functional protein, in the case of DOPE/DOPG (3:1).

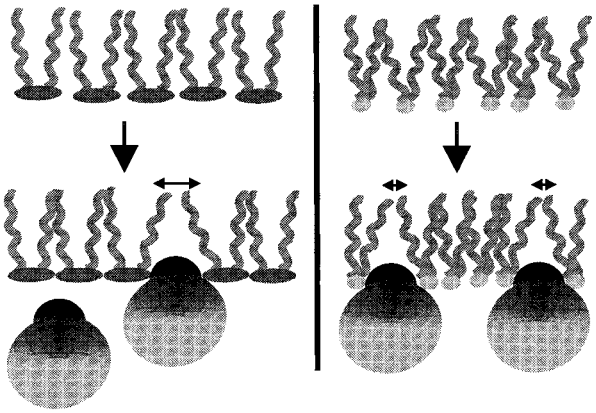

FIGURE 7: Model to explain the differences between bilayer and nonbilayer lipids in the calculated areas of the inserted domain of Δ2−75. The left panel shows a monolayer of bilayer lipids, which have an overall cylindrical shape. The right panel shows a monolayer of nonbilayer lipids, which have an overall conical shape. In this monolayer there is much more free space between the lipid headgroups. Due to their shape, the lipids have to move apart from each other much more in the case of bilayer lipids compared to nonbilayer lipids, to let the protein insert to the same depth. As a result a lower area of the inserted domain is determined in the case of nonbilayer lipids.

pressure−area isotherms that were used for the calculation of the protein/lipid ratios (*P*/*L*). It can be seen in Figure 6 that the apparent area per protein molecule decreases from 1781 ± 195 Å^2 for DGDG to 196 ± 38 Å^2 for DOG. These numbers are reasonable when compared to the size of the protein, which is known from the crystal structure. According to Paetzel et al. (*16*), the Δ2−75 molecule has an overall conical shape with dimensions of roughly 60 × 40 × 70 Å, which gives a cross-sectional area between 2400 and 4200 Å^2. Figure 6 shows that, although Δ2−75 has a higher affinity for nonbilayer lipids, the size of the inserted domain of the protein appears much smaller for nonbilayer lipids compared to bilayer lipids.

A model to explain this striking result is given in Figure 7. The surface pressure that is measured in a monolayer experiment is determined by that part of the molecule that occupies the largest area and thus results in the highest pressure. In the case of lipid molecules, especially nonbilayer lipids with a small headgroup area compared to the area of the acyl chains, there can be free space between the lipid headgroups, while there exists a certain pressure between the acyl chains. When a protein inserts between the lipid headgroups, the lipids do not have to move so far apart from each other in the case of nonbilayer lipids. As a result a lower area of the inserted domain of Δ2−75 would be determined, even if the area of the inserted domain is the same.

If we assume that the mode of insertion of Δ2−75 is similar for all used lipid systems, it is possible to calculate the amount of free space present between the lipid headgroups, relative to DOPC, by

$$A_{\mathrm{free,l}} = (P/L)[(A_{\mathrm{p,app}})_{\mathrm{DOPC}} - (A_{\mathrm{p,app}})_{\mathrm{l}}] \qquad (3)$$

where $A_{\mathrm{free,l}}$ is the free area per lipid molecule in the headgroup region, relative to DOPC, and *P*/*L* is the protein/lipid ratio. $(A_{\mathrm{p,app}})_{\mathrm{l}}$ and $(A_{\mathrm{p,app}})_{\mathrm{DOPC}}$ are the calculated apparent size of the inserted domain of the protein for the lipids L and DOPC, respectively. Figure 8 shows that with these assumptions the free space between the lipid headgroups is largest for DOPE, for which it is 15 ± 7 Å^2 relative to DOPC. Although this calculation was done for lipid monolayers, these results are probably also valid for lipid bilayers, because the lipid packing at the final surface pressures of the monolayer experiments (28−35 mN/m) is comparable to the packing in a lipid bilayer, which corresponds to a surface pressure of 30−35 mN/m (*26*).

How do these calculated numbers for the free space between the lipid headgroup compare to known values from literature? Experimentally, it is possible to determine the effective size of a lipid molecule, but it is very difficult to determine the size of the headgroup and acyl chains separately. However, it is possible to calculate the cross-sectional area of the lipid headgroup (a_{h}) and tails (a_{t}) based on computer simulations, which was done by Huang et al. (*30*) for DOPE and DOPC (simulated as a hydrated bilayer at 37 °C). They calculated an area of the tails of 71.0 Å^2 for DOPC and 70.4 Å^2 for DOPE. For the lipid headgroups they calculated an area of 70.6 Å^2 for DOPC, while this was only 58.2 Å^2 for DOPE. This results in a free space in the headgroup region of ±12 Å^2 per lipid molecule for DOPE relative to DOPC. Our value of 15 Å^2/lipid molecule is in fair agreement with the results of this computer simulation.

To study the effect of nonbilayer lipids on membrane insertion of proteins, we used Δ2−75 as a model protein. What is the biological relevance of these findings for the protein itself? Leader peptidase cleaves off signal peptides of precursor proteins during or after translocation. During translocation the N-terminus of the signal peptide stays at

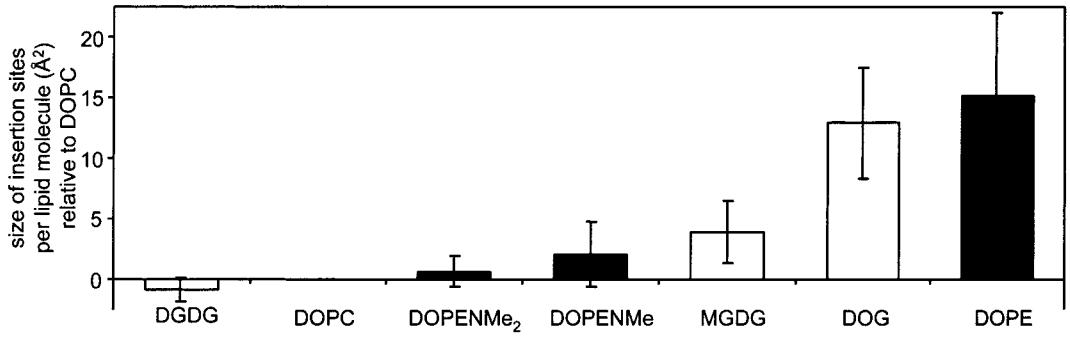

FIGURE 8: Calculated size of the insertion sites (Å^2/lipid molecule) relative to DOPC, based on the calculated area of the inserted domain of Δ2−75 as shown in Figure 6 and the model as shown in Figure 7. This was done for PE and its *N*-methylated derivatives (black bars), as well as for the other (non)bilayer lipids DGDG, MGDG, and DOG (white bars). The formula used for the calculation is given in the Discussion. Error bars represent the standard deviation.

the cytosolic site of the membrane (*31*, *32*), while its hydrophobic core is probably too short to span the membrane. The crystal structure of the catalytic domain of leader peptidase shows that the exposed hydrophobic patch across the molecule, which is assumed to be the membrane association surface, includes the substrate binding site and catalytic center (*16*). It was suggested that the protein inserts this domain into the membrane lipid bilayer, presumably optimizing contact with the signal-peptide cleavage site. The abundant presence of nonbilayer lipid PE (75% of the total lipid composition of *Escherichia coli*) would facilitate the insertion of this catalytic domain of leader peptidase into the membrane, and thus the cleavage of the signal peptide.

REFERENCES

1. Cullis, P. R., and de Kruijff, B. (1979) *Biochim Biophys Acta 559*, 399−420.
2. Israelachvili, J. N., Marcelja, S., and Horn, R. G. (1980) *Q. Rev. Biophys. 13*, 121−200.
3. Karlsson, O. P., Rytomaa, M., Dahlqvist, A., Kinnunen, P. K., and Wieslander, A. (1996) *Biochemistry 35*, 10094−102.
4. Goldfine, H., Rosenthal, J. J., and Johnston, N. C. (1987) *Biochim. Biophys. Acta 904*, 283−9.
5. Morein, S., Andersson, A., Rilfors, L., and Lindblom, G. (1996) *J. Biol. Chem. 271*, 6801−9.
6. Rietveld, A. G., Killian, J. A., Dowhan, W., and de Kruijff, B. (1993) *J. Biol. Chem. 268*, 12427−33.
7. Rietveld, A. G., Chupin, V. V., Koorengevel, M. C., Wienk, H. L., Dowhan, W., and de Kruijff, B. (1994) *J. Biol. Chem. 269*, 28670−5.
8. Keller, S. L., Bezrukov, S. M., Gruner, S. M., Tate, M. W., Vodyanoy, I., and Parsegian, V. A. (1993) *Biophys. J. 65*, 23−7.
9. Rietveld, A. G., Koorengevel, M. C., and de Kruijff, B. (1995) *EMBO J. 14*, 5506−13.
10. van der Does, C., Swaving, J., van Klompenburg, W., and Driessen, A. J. (2000) *J. Biol. Chem. 275*, 2472−8.
11. Vikstrom, S., Li, L., Karlsson, O. P., and Wieslander, A. (1999) *Biochemistry 38*, 5511−20.
12. Senisterra, G., and Epand, R. M. (1993) *Arch Biochem. Biophys. 300*, 378−83.
13. Attard, G. S., Templer, R. H., Smith, W. S., Hunt, A. N., and Jackowski, S. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 9032−6.
14. Gruner, S. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3665−9.
15. van Klompenburg, W., Paetzel, M., de Jong, J. M., Dalbey, R. E., Demel, R. A., von Heijne, G., and de Kruijff, B. (1998) *FEBS Lett. 431*, 75−9.
16. Paetzel, M., Dalbey, R. E., and Strynadka, N. C. (1998) *Nature 396*, 186−90.
17. Hunter, G. W., Negash, S., and Squier, T. C. (1999) *Biochemistry 38*, 1356−64.
18. Ahn, T., Oh, D. B., Lee, B. C., and Yun, C. H. (2000) *Biochemistry 39*, 10147−53.
19. Bogdanov, M., Umeda, M., and Dowhan, W. (1999) *J. Biol. Chem. 274*, 12339−45.
20. Nouwen, N., Tommassen, J., and de Kruijff, B. (1994) *J. Biol. Chem. 269*, 16029−33.
21. Kuo, D. W., Chan, H. K., Wilson, C. J., Griffin, P. R., Williams, H., and Knight, W. B. (1993) *Arch. Biochem. Biophys. 303*, 274−280.
22. Paetzel, M., Chernaia, M., Strynadka, N., Tschantz, W., Cao, G., Dalbey, R. E., and James, M. N. (1995) *Proteins: Struct., Funct., Genet. 23*, 122−5.
23. van Klompenburg, W., Whitley, P., Diemel, R., von Heijne, G., and de Kruijff, B. (1995) *Mol. Membr. Biol. 12*, 349−53.
24. Tschantz, W. R., Paetzel, M., Cao, G., Suciu, D., Inouye, M., and Dalbey, R. E. (1995) *Biochemistry 34*, 3935−41.
25. Dottavio-Martin, D., and Ravel, J. M. (1978) *Anal. Biochem. 87*, 562−5.
26. Demel, R. A. (1994) *Subcell. Biochem. 23*, 83−120.
27. Rietsch, J., Pattus, F., Desnuelle, P., and Verger, R. (1977) *J. Biol. Chem. 252*, 4313−8.
28. Hope, M. J., Bally, M. B., Webb, G., and Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55−65.
29. Rouser, G., Fkeischer, S., and Yamamoto, A. (1970) *Lipids 5*, 494−6.
30. Huang, P., Perez, J. J., and Loew, G. H. (1994) *J. Biomol. Struct. Dyn. 11*, 927−56.
31. Kuhn, A. (1987) *Science 238*, 1413−5.
32. Shaw, A. S., Rottier, P. J., and Rose, J. K. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7592−6.

BI002903A