Evidence for Phospholipid Microdomain Formation in Liquid Crystalline Liposomes Reconstituted with *Escherichia coli* Lactose Permease

Jukka Y. A. Lehtonen and Paavo K. J. Kinnunen
Biomembrane Research Group, Department of Medical Chemistry, Institute of Biomedicine, University of Helsinki, FIN-00014 Helsinki, Finland

ABSTRACT The well-characterized integral membrane protein lactose (lac) permease from *Escherichia coli* was reconstituted together with trace amounts (molar fraction $X = 0.005$ of the total phospholipid) of different pyrene-labeled phospholipid analogs into 1-palmitoyl-2-oleoyl-sn-glycero-3-sn-glycero-3-phospho-*rac'*-glycerol (POPG) liposomes. Effects of lac permease on bilayer lipid dynamics were investigated by measuring the excimer-to-monomer fluorescence intensity ratio I_E/I_M. Compared to control vesicles, the presence of lac permease (at a protein:phospholipid stoichiometry *P/L* of 1:4.000) increased the rate of excimer formation by 1-palmitoyl-2[6-(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine (PPDPC) by approximately fivefold. Decreasing *P/L* from approximately 1:4.000 to 1:7.600 decreased the I_E/I_M for PPDPC from 0.16 to 0.05, respectively. An increase in bilayer fluidity due to permease is unlikely, thus implying that the augmented I_E/I_M should arise from partial lateral segregation of PPDPC in the vesicles. This notion is supported by the further 38% increase in I_E/I_M observed for the pyrene-labeled Cys-148 lac permease reconstituted into POPG vesicles at *P/L* 1:4000. The importance of the length of the lipid-protein boundary is implicated by the reduction in I_E/I_M resulting from the aggregation of the lac permease in vesicles by a monoclonal antibody. Interestingly, excimer formation by 1-palmitoyl-2[6-(pyren-1-yl)]hexanoyl-sn-glycero-3-phosphocholine (PPHPC) was enhanced only fourfold in the presence of lac permease. Results obtained with the corresponding pyrenyl phosphatidylglycerols and -methanols were qualitatively similar to those above, thus indicating that lipid headgroup-protein interactions are not involved. Inclusion of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamino-*N*-(5-fluoresceinthio-carbamoyl) (DPPF, $X = 0.005$) into reconstituted lactose permease vesicles containing PPDPC caused a nearly 90% decrease in excimer fluorescence, whereas in control vesicles lacking the reconstituted protein only 40% quenching was evident. The addition of 1,2-dipalmitoyl-sn-glycero-3-phospho-*rac'*-glycerol (DPPG) decreased I_E/I_M for PPDPC, revealing the driving force for the lateral segregation of this probe to become attenuated. More specifically, for protein-free bilayers at $X_{DPPG} = 0.10$ the rate of lateral diffusion of PPDPC in POPG is diminished, as evidenced by the 24% decrement in I_E/I_M, under these conditions the increase in I_E/I_M due to lac permease was strongly reduced, by ~84%. The present data are interpreted in terms of the hydrophobic mismatch theory, which predicts that integral membrane proteins will draw lipids of similar hydrophobic thickness into their vicinity. In brief, the approximate lengths of most of the predicted 12 hydrophobic, membrane-spanning α-helical segments of lactose permease range between 28.5 and 37.5 Å and thus exceed the hydrophobic thickness of POPG of ~25.8 Å. Therefore, to reduce the free energy of the assembly, longer lipids such as PPDPC and DPPF are accumulated in the immediate vicinity of lactose permease in fluid, liquid crystalline POPG bilayers.

INTRODUCTION

A large body of evidence indicates lateral heterogeneity of lipid distribution to be common in biological membranes (for reviews see, e.g., Kinnunen, 1991; Welti and Glaser, 1994; Bergelson, 1995). Major emphasis has been in the elucidation of principles governing organization in fluid bilayers, as this is the state characterizing most, if not all, functionally active biomembranes. Importantly, some of the mechanisms responsible for the formation and maintenance of membrane lateral heterogeneity are now beginning to be understood. Phase segregation in proper mixtures can be caused by ethanol (Rowe, 1987). Osmotically induced domain formation was recently demonstrated in fluid membranes (Lehtonen and Kinnunen, 1995). Density fluctuations at the main transition have been proposed to cause dynamic lateral clusters (Mouritsen and Jørgensen, 1994) and are supported by the recent results by Pedersen et al. (1996). Segregation has been shown for dimyristoyl- and distearoylphosphatidylcholine mixtures above their phase transition temperature (Melchior, 1986). Likewise, nonideal mixing of 16:0, 18:1 PS and di-12:1 PC has been detected (Huang et al., 1993; Hinderliter et al., 1994), and Raman spectroscopy studies also provide evidence for microscopic domain formation in unsaturated PC membranes (Litman et al., 1991). Formation of lipid domains in liquid crystalline bilayers, resulting from the mismatch of the effective lengths of the hydrophobic parts of the constituent phospholipids, has recently been described (Lehtonen et al., 1996). To this end, there is experimental evidence revealing organization of fluid binary lipid mixtures into regular superlattice arrays (Berclaz and McConnell, 1981; Somerharju et al., 1985; Kinnunen et al., 1987; Tang and Chong, 1992; Chong et al., 1994; Tang et al., 1995).

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Address reprint requests to Dr. Paavo K. J. Kinnunen, Department of Medical Chemistry, Institute of Biomedicine, P.O. Box 8, FIN-00014 University of Helsinki, Helsinki, Finland. Tel.: 358-9-1918237; Fax: 358-9-1918276; E-mail: paavo.kinnunen@helsinki.fi.

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Ordering in fluid bilayers may also arise because of proteins. For peripheral proteins this is exemplified by, for example, prothrombin (Mayer and Nelsestuen, 1981), cytochrome *c* (Mustonen et al., 1987), protein kinase C (Yang and Glaser, 1995), histone H1 (Rytömaa and Kinnunen, 1996), and annexins (Junker and Creutz, 1993), all of which cause clustering of acidic phospholipids. The requirement for domains enriched in acidic phospholipids has been verified for the membrane association of cytochrome *c* (Mustonen et al., 1987) and gelsolin (Janmey and Chaponnier, 1995). Furthermore, integral, membrane-spanning proteins have both physical and chemical requirements for their interactions with lipids, and they exhibit selectivity with respect to both the hydrophobic part and the headgroup. Electrostatic attraction of acidic phospholipids to the basic residues adjacent to the transmembrane segment of integral membrane proteins has been described for Ca^{2+}-ATPase as well as the K^+ channel (Verbist et al., 1993; Horváth et al., 1995).

The *Escherichia coli* inner membrane integral protein lactose permease is exceptionally well characterized (Kaback, 1986, 1987, 1992; Kaback et al., 1994; Frilingos et al., 1994; Wu et al., 1995). Based on circular dichroism measurements and its amino acid sequence, a model for secondary structure has been suggested in which permease spans the membrane with 12 α-helical hydrophobic stretches separated by hydrophilic domains (Kaback et al., 1994). The combined use of site-directed mutagenesis and site-directed fluorescence labeling has led to a model describing the proximity relationships of some of the membrane-spanning helices (Jung et al., 1993, 1994a; Kaback et al., 1994).

Lactose permease (lac permease) has not been reported to possess any particular requirements for the acyl chain structure of phospholipids. It seems, however, that its transport function in cells is impaired below the phase transition temperature of the lipids (Thilo et al., 1977). As for the phospholipid polar headgroup, lac permease has a special requirement for the full activity of an amino phospholipid; i.e., either phosphatidylethanolamine or phosphatidylserine appears to be needed (Chen and Wilson, 1984; Bogdanov and Dowhan, 1995). In vivo studies have shown that lactose permease distributes preferentially in fluid lipid domains (Thilo et al., 1977). The influence of lac permease on the dynamics of lipids has not been investigated.

Pyrene-labeled phospholipids have become widely accepted tools for studying the properties of biomembranes and membrane models (for a brief review, see Kinnunen et al., 1993). The popularity of this type of fluorescent probes rests mainly in the particular photophysics of pyrene, as follows. A monomeric excited-state pyrene relaxes radiatively to ground state by emitting photons with a maximum at ~394 nm (I_M), the exact peak energy and spectral fine structure depending on solvent polarity. During its lifetime, the excited-state pyrene may form a characteristic short-lived complex excimer (excited dimer) with a ground-state pyrene. This complex relaxes back to two ground-state pyrenes by emitting quanta as a broad and featureless band centered at ~480 nm (I_E). In the absence of possible quantum mechanical effects (Kinnunen et al., 1987) and the formation of superlattices, the excimer-to-monomer fluorescence intensity ratio (I_E/I_M) depends on the rate of collisions between pyrenes (Förster, 1969). Consequently, for a pyrene-containing phospholipid analog such as 1-palmitoyl-2[6-(pyren-1-yl)]decanoyl-sn-glycero-3-phosphocholine (PPDPC), the value for I_E/I_M reflects the lateral mobility (Galla and Sackmann, 1974; Galla et al., 1979) as well as the local concentration of the fluorophore in the membrane (Galla and Hartmann, 1980; Somerharju et al., 1985; Hrésko et al., 1986; Eklund et al., 1988).

We investigated the influence of lac permease on lipid dynamics in reconstituted liposomes as revealed by different pyrene-labeled phospholipids. Our results suggest that these fluorescent lipids become enriched into domains in 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-rac′-glycerol (POPG) vesicles containing lac permease. The extent of lateral enrichment of the pyrene-labeled phospholipids depends on their effective acyl chain length, the average thickness of the bilayer, as well as the protein:lipid molar ratio.

MATERIALS AND METHODS

Materials

POPG was purchased from Avanti Polar Lipids (Alabaster, AL), and the pyrene lipids were from K&V Bioware (Espoo, Finland). The purity of lipids was checked by thin-layer chromatography on plates coated with silicic acid (Merck, Darmstadt, Germany), using a chloroform/methanol/water (65:25:4, v/v) solvent system, and examination of the plates for pyrene fluorescence or after iodine staining. Monoclonal antibody 4B1 was from BabCo (Richmond, CA). Pyrene maleimide was from Molecular Probes (Eugene, OR). The concentrations of the phospholipids were determined by phosphorus assay (Bartlett, 1959), and those of the pyrenyl probes were determined spectrophotometrically at 342 nm, using 42,000 cm^{-1} as the molar extinction coefficient for pyrene.

Bacterial strains and plasmids

E. coli T184 [lacI$^+$O$^+$Z$^-$Y$^-$(A), rpsL, met$^-$, thr$^-$, recA, hsdM, hsdR/F′, and lacI^{q}O$^+$Z^{D118}(Y$^+$A$^+$)] (Teather et al., 1980) harboring plasmid pT7-5/lacY-L6XB (Consler et al., 1993), which encodes wild-type permease and a biotin acceptor domain in the middle cytoplasmic loop, was used for overexpression because of induction with isopropyl 1-thio-β-D-galactopyranoside.

Oligonucleotide-directed site-specific mutagenesis

The plasmid used to express the Cys-148 mutant was generously provided by Drs. K. Jung and H. R. Kaback (Howard Hughes Medical Institute, Molecular Biology Institute, University of California, Los Angeles). Introduction of Cys into position 148 instead of Ser was performed by oligonucleotide-directed, site-specific mutagenesis of the cassette *lacY* gene in the plasmid pT7-5 encoding cysteineless lac permease (van Iwaarden et al., 1991; Jung et al., 1993, 1995). The polymerase chain reaction (PCR) overlap extension method (Ho et al., 1989) was employed to create the mutation. PCR products were purified in agarose gels, gene cleaned, and digested with *Bss*HII and *Xho*I restriction endonucleases. The *Bss*HII-*Xho*I

fragments were isolated from low-melting-point agarose gels and ligated to similarly treated pT7-5/cassette *lacY* vector.

Expression and purification of biotinylated lac permease

Six liters of cells were cultivated at 37°C, and permease production was induced with 0.3 mM isopropyl 1-thio-β-D-galactopyranoside. Membranes were prepared as described (Viitanen et al., 1986) and extracted with 1.25% octyl β-D-glucopyranoside. Wild-type permease with biotin acceptor domain was purified by affinity chromatography on immobilized monomeric avidin (Pierce, Rockford, IL), as described by Consler et al. (1993). The resin was equilibrated with 50 potassium phosphate (pH 7.4)/150 mM KCl/1.25% octyl β-D-glucopyranoside (w/v)/1 mM dithiothreitol/20 mM lactose/0.125 mg/ml POPG. After application of the sample, the column was washed thoroughly with column buffer. Bound lac permease was then eluted with 8 mM *d*-biotin in column buffer without dithiothreitol. The isolated protein was pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Laemmli, 1970), followed by silver as well as by Coomassie blue staining.

Labeling of Cys-148 *lac* permease

Purified Cys-148 mutant was labeled with pyrene maleimide (Jung et al., 1993). This reagent was dissolved in *N,N*-dimethylformamide and slowly added to a stirred sample of purified lac permease until a 10-fold molar excess to protein was achieved. The reaction mixture was incubated for 4 h at 4°C in the dark with stirring and subsequently centrifuged at 15,000 × g_{max} for 20 min to remove undissolved reagent. Thereafter, a fivefold excess of glutathione over pyrene maleimide was added, and the sample was incubated for 1 h to quench the reaction.

Reconstitution of lactose permease into liposomes

Fractions containing purified permease were pooled, and the protein was reconstituted by the detergent dilution procedure (Viitanen et al., 1986) with POPG to yield a protein-to-lipid (*P/L*) stoichiometry of 1:4000. Liposomes with permease were centrifuged (150,000 × g_{max}) and resuspended in 50 mM potassium phosphate (KP_i) (pH 7.5), followed by five freeze-thaw/sonication cycles. Lipid concentrations of the reconstituted liposomes were determined by phosphorus assay (Bartlett, 1959). A series of reconstituted liposomes was made so as to contain varying amounts of permease, while the molar fraction *X* of the pyrene phospholipid analogs was maintained constant at 0.005. More specifically, the *P/L* ratio was decreased by adding appropriate amounts of both POPG and the fluorescent lipids in a small volume of ethanol (max. 15 μl/ml) followed by up to five freeze/thaw sonication cycles, until no further change in the I_E/I_M was observed.

Fluorescence measurements

Fluorescence emission spectra were recorded using a Perkin-Elmer LS50 spectrofluorometer with a magnetically stirred, thermostated cuvette compartment. Temperature was controlled with a Lauda RC6 (Lauda, Lauda-Königshafen, Germany) circulating waterbath. The instrument is connected to a 50-MHz 486 computer, and the data were analyzed by the dedicated software from Perkin-Elmer. To monitor changes in the pyrene-lipid dynamics, the ratio of intensities at 470 nm and 378 nm was measured. Bandpasses of 4 nm were used both for the excitation and emission beams, respectively. For more facile experimentation, the fluorescence data were collected in the presence of atmospheric oxygen (Mustonen and Kinnunen, 1993; Mustonen et al., 1993). Intensities shown were not corrected for instrument response.

Resonance energy transfer measurements

Electronic excitation energy can be efficiently transferred between a fluorescent energy donor and a suitable energy acceptor over distances as large as 60 Å, the rate of the transfer depending on the reciprocal of the sixth power of the average distance between the donor and the acceptor. In addition, orientation of the acceptor and donor excitation and relaxation dipoles, respectively, as well as the magnitude of the spectral overlap integral determine the efficiency of this process (Stryer, 1978). The strong spectral overlap of pyrene excimer emission and fluorescein absorbance allows for efficient resonance energy transfer (Kõiv et al., 1995). Notably, the r^{-6} dependency of the dipole-dipole coupling efficiency does not apply to the 2-D case (Drake et al., 1991), for instance, when the probes reside in liposomes. Accordingly, quantitative analysis of data such as that described here is ambigous. Despite this limitation, this method makes it possible to obtain an estimate of the colocalization of the fluorescein labeled lipid 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamino-*N*-(5-fluoresceinthiocarbamoyl) (DPPF) with domains enriched in the pyrene-labeled lipid, PPDPC (Lehtonen et al., 1996). For this purpose PPDPC and DPPC were simultanously introduced into liposomes as described above, whereafter fluorescence spectra were recorded so as to obtain the extent of quenching of pyrene excimer emission by DPPF in both the absence and presence of lac permease.

RESULTS

Influence of lac permease on pyrene-lipid excimer formation in liposomes

As stated above, for a pyrene-containing phospholipid analog such as PPDPC, the value for I_E/I_M depends on the rate of intermolecular collisions between pyrenes and thus reflects the lateral mobility as well as the local concentration of the fluorophore in the membrane (for a brief review, see Kinnunen et al., 1993). To investigate the influence of the integral membrane protein lac permease on lipid dynamics in liposomes, we reconstituted this protein into POPG liposomes together with different pyrene-labeled phospholipid analogs, and protein-free liposomes served as controls. For PPDPC ($X = 0.005$) residing in POPG liposomes, an I_E/I_M of 0.03 was measured at 25°C (Fig. 1). At this temperature POPG is in a fluid, liquid crystalline state, and except for slight enrichment of the probes due to hydrophobic mismatch (Lehtonen et al., 1996), no significant clustering of PPDPC is anticipated. In keeping with the above, there are no discontinuities in the I_E/I_M versus temperature scans (Fig. 2 *A*), which only reveal a monotonous increase in I_E/I_M due to thermally augmented lateral diffusion of the fluorescent probe. The inclusion of lac permease into liposomes at a protein:lipid molar ratio of *P/L* of 1:4000 resulted in an approximately fivefold increase (from 0.03 to 0.16) in the rate of excimer formation by PPDPC at 25°C (Fig. 2 *A*). The enhancement of excimer formation by PPDPC could result either from an augmented lateral mobility of the probe or from its topical enrichment in the presence of permease. For POPG bilayers increase in fluidity due to the protein is unlikely, thus favoring the possibility that lateral segregation of PPDPC takes place in the presence of permease.

Interestingly, I_E/I_M is strongly dependent on *P/L*, and the rate of excimer formation increases from 0.06 to 0.20 upon increasing the content of lac permease in liposomes, with

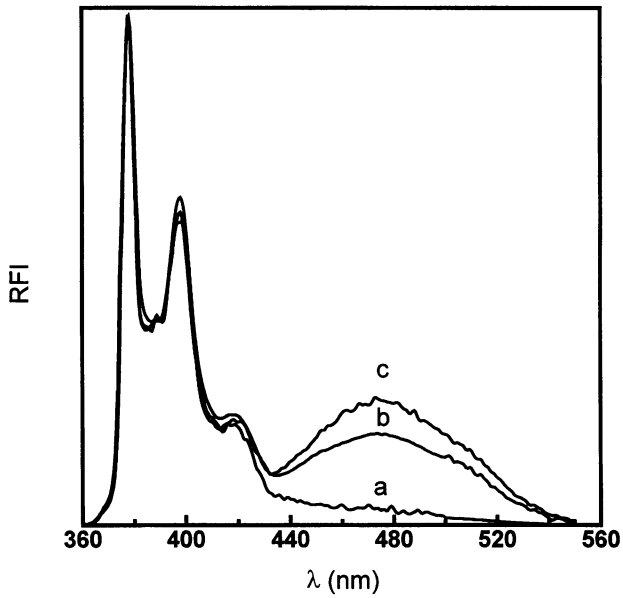

FIGURE 1 Fluorescence emission spectra for PPDPC (X = 0.005) in POPG as such (*a*) and with lac permease (1:4.000, protein:lipid molar ratio, *P/L*), (*b*). Spectrum for lactose permease mutant Cys-148 prepared by site-directed mutagenesis and subsequently labeled with pyrene maleimide reconstituted into similar vesicles containing PPDPC at a protein:lipid molar ratio *P/L* = 1:4.000 is also shown. (*c*) Medium was 50 mM phosphate buffer, pH 7.4, and temperature 25°C. Spectra were normalized for monomer emission at 378 nm.

P/L increasing from 1:7.600 to 1:4.000 (Fig. 3). The lack of aggregation verified in a freeze-fracture study on reconstituted lactose permease can be anticipated to be opposed by electrostatic repulsion due to the net positive charge of ~11 of this protein (Carrasco et al., 1984; Costello et al., 1984; Sun et al., 1996). Monoclonal antibody 4B1 has been reported to cause reconstituted permease to aggregate (Carrasco et al., 1984; Costello et al., 1984; Sun et al., 1996). The addition of a threefold molar excess (with respect to permease) of this antibody decreased I_E/I_M (Fig. 3). The relative decrement in I_E/I_M due to the antibody was independent of *P/L* and was 40% over the *P/L* range studied. Assuming random orientation of the reconstituted lac permease in the vesicles, approximately half of the protein should be accessible to the added antibody, in keeping with the observed extent of reduction in I_E/I_M. Notably, the *P/L* dependency and the effect of aggregation on I_E/I_M both indicate the length of the protein-lipid boundary in the vesicles to be proportional to the enhancement in the rate of excimer formation observed in the presence of lac permease.

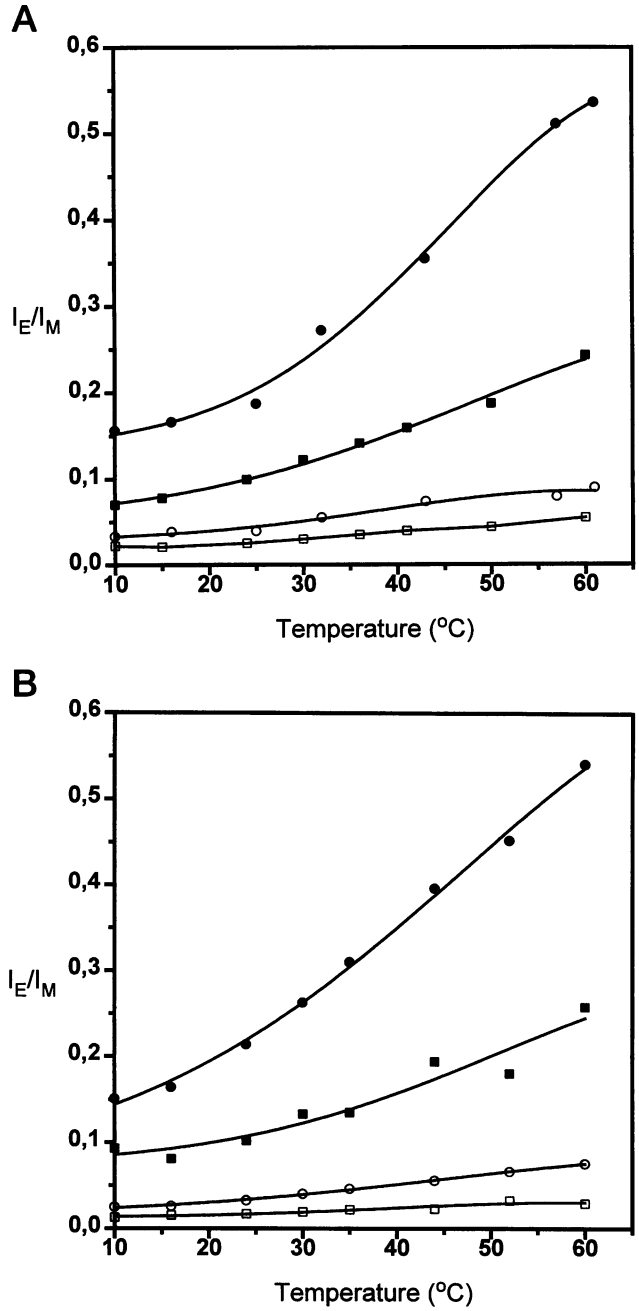

FIGURE 2 (*A*) I_E/I_M versus temperature scans for PPDPC (X = 0.005, ●, ○) and PPHPC (X = 0.005, ■, □) labeled POPG control vesicles (○, □) or liposomes containing lactose permease at a protein:lipid molar ratio *P/L* of 1:4.000 (●, ■). (*B*) An experiment similar to that in *A*, but using either PPDPG (●, ○) or PPHPG (■, □) as probes, both in the absence (○, □) and presence (●, ■) of lactose permease. The mole fraction of the probes was 0.005. The aqueous medium was 50 mM phosphate buffer at pH 7.4, and experiments were carried out at 25°C.

Evidence for the enrichment of PPDPC due to lactose permease

Cysteineless lactose permease mutants harboring Cys-148 were constructed and labeled with pyrene maleimide, which specifically reacts with the sulfhydryl moiety. This mutant was chosen because residue 148 has been shown to be exposed to the lipid phase (Mitaku et al., 1984; for a mutagenesis study see Jung et al., 1994b). The pyrene-labeled permease was reconstituted into POPG vesicles containing PPDPC (X = 0.005) at a *P/L* of 1:4.000. Compared to control vesicles reconstituted with unlabeled lac permease, a further increase by 38% in I_E/I_M due to pyrene-

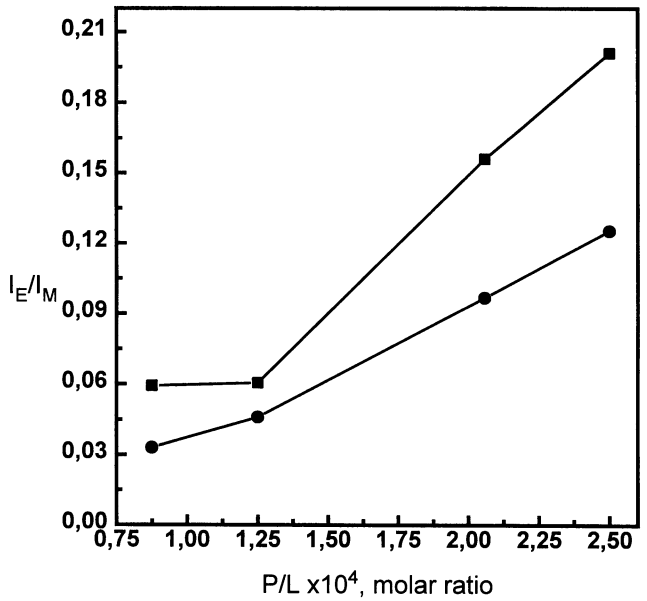

FIGURE 3 Effect of varying permease:lipid molar ratio *P/L* on the excimer formation by PPDPC (■). The other curve represents the effect of a threefold molar excess (with respect to lac permease) of monoclonal antibody 4B1 (●). Otherwise the conditions were as decribed for Fig. 1.

labeled lac permease was evident (Fig. 1). Vesicles with labeled lactose lactose permease and containing no PPDPC did show neglible excimer fluorescence. Importantly, the observed increase in I_E/I_M is thus unproportionally high, as the amount of pyrene added to the labeled protein corresponds only to a 5% increase in X_{PPDPC}, i.e., from 0.005 to 0.00525. For this increase in X_{PPDPC}, the corresponding enhancement in I_E/I_M would be <20%. The increase in excimer formation in membranes with pyrene-labeled lac permease mutant confirms the pyrene moiety in protein to be accessible to collisions with pyrene-labeled phospholipids. Furthermore, these data suggest PPDPC to be enriched in the vicinity of the pyrene label at Cys-148.

The influence of pyrene-lipid structure

To investigate the dependency of the effect of lac permease on the effective length of the probe, we subsequently utilized, instead of PPDPC, another pyrene-labeled lipid analog, 1-palmitoyl-2[6-(pyren-1-yl)]hexanoyl-sn-glycero-3-phosphocholine (PPHPC), in which the pyrene-containing chain is shorter by four methyl segments. Compared to control liposomes, the presence of permease enhances excimer formation by PPHPC by approximately fourfold (Fig. 2 *A*). The increase in excimer formation by PPHPC due to lac permease is ~50% of that observed for PPDPC. The thermal enhancement in excimer formation by PPHPC and PPDPC is higher in vesicles containing permease (Fig. 2 *A*). An increase in temperature from 10°C to 60°C results in a 2.5-fold increase in the I_E/I_M for POPG vesicles containing PPHPC, whereas in the presence of lac permease (*P/L* = 1:4.000), a 3.3-fold increase was evident. The temperature-induced increase in the I_E/I_M for PPDPC in the presence of the protein is 3.7-fold, i.e., higher than for PPHPC, thus indicating that upon an increase in temperature PPDPC becomes more efficiently enriched than PPHPC. Alternatively, the lateral diffusion of PPDPC could be faster than for PPHPC. These possibilities are not mutually exclusive.

To elucidate the possible effects of the headgroup structure, the above experiments were repeated, using instead of the phosphatidylcholines the corresponding pyrenyl phosphatidylglycerols as well as phosphatidylmethanols, 1-palmitoyl-2[6-(pyren-1-yl)]hexanoyl-sn-glycero-3-phospho-rac′-glycerol (PPHPG), and 1-palmitoyl-2[6-(pyren-1-yl)]decanoyl-sn-glycero-3-phospho-rac′-glycerol (PPDPG), as well as PPHPM and PPDPM, respectively. Results qualitatively similar to those described above were obtained with these negatively charged probes (Fig. 2 *B*). In brief, the I_E/I_M ratios for PPDPG and PPDPM were higher than those for PPHPG and PPHPM. These data reveal that the segregation of pyrene lipids is strongly influenced by the structure of the hydrophobic chains, whereas the headgroup interactions do not appear to be involved. Furthermore, upon a decrease in the thickness of the POPG bilayer caused by the increase in temperature, the driving force for the lateral enrichment of PPDPC can be anticipated to be stronger than for the shorter lipid PPHPC. Accordingly, the difference between these two probes would be compatible with hydrophobic mismatch, causing the enrichment of the pyrene-labeled lipids in the presence of lac permease.

Further evidence for microdomain formation

As concluded above, compared to PPHPC, -PG, and -PM (containing pyrenehexanoyl chains), the probes with larger effective lengths, i.e., PPDPC, -PG, and -PM (with pyrenedecanoyl chains), appear to become more efficiently enriched in the presence of lac permease.

Increasing the content of DSPC in binary alloys with DMPC has been demonstrated to cause a linear increase in bilayer thickness (Sankaram and Thompson, 1992). To test the effect of the inclusion of a thicker lipid on the enrichment of PPDPC due to the presence of lac permease in POPG vesicles, we also included 1,2-dipalmitoyl-sn-glycero-3-phospho-*rac′*-glycerol (DPPG) (X = 0.10) in the POPG matrix. In keeping with hydrophobic mismatch providing the driving force for the enrichment of PPDPC in the presence of lac permease, the inclusion of DPPG resulted in a decrease in I_E/I_M. Interestingly, for protein-free bilayers at X_{DPPG} = 0.10, the rate of lateral diffusion of PPDPC in POPG is slightly attenuated, as evidenced by the 24% decrease in I_E/I_M. Importantly, in the presence of DPPG, the increase in I_E/I_M due to lac permease was strongly reduced, by ~84% (Table 1).

Further evidence for the segregation of PPDPC in the presence of permease was provided by experiments in which DPPF was included together with PPDPC in the liposomes (Table 1). Spectral overlap of pyrene excimer

TABLE 1 Comparison of the data for DPPG and DPPF incorporated into PPDPC/POPG liposomes either lacking or containing lac permease (*P/L* = 1:4000)

	Parameter measured			Δ%
		X_{DPPG}		
		0	0.10	
Control	I_E/I_M	0.030	0.0228	−24.0
Lac permease	I_E/I_M	0.160	0.0257	−83.9
		X_{DPPF}		
		0	0.005	
Control	I_{480}	3.86 (1.00)	2.29 (0.59)	−40.5
Lac permease	I_{480}	44.4 (11.50)	5.96 (1.54)	−86.5

I_E/I_M for PPDPC is shown in both the presence and absence of DPPG. I_{480} is the excimer fluorescence intensity measured in the presence or absence of DPPF. To better emphasize the latter set of data, normalized fluorescence intensity values are also provided (in brackets). More specifically, the emission intensity for control vesicles was taken as 1, and the rest of the data were normalized with respect to this. See text for details.

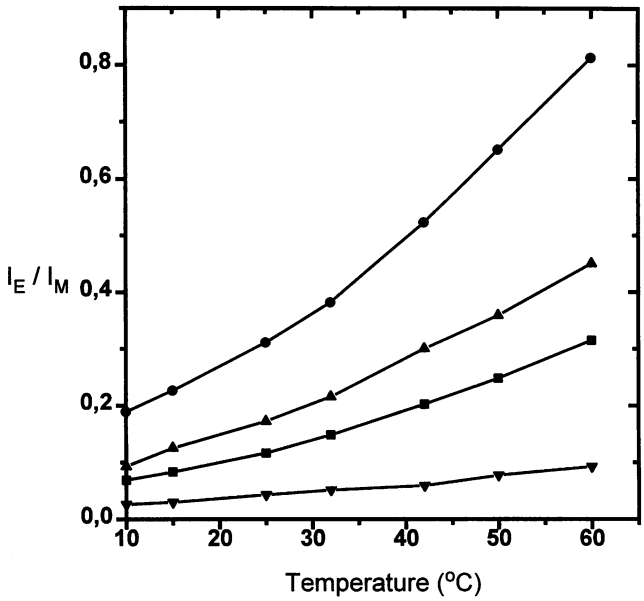

FIGURE 4 Estimation of the relative enrichment of PPDPC in POPG vesicles due to the presence of lactose permease. I_E/I_M was measured as a function of temperature for vesicles without lac permease. X_{PPDPC} was 0.001 (▼), 0.003 (■), 0.005 (▲), and 0.006 (●).

emission and fluorescein allows for efficient fluorescence energy transfer between pyrene lipids and DPPF (Kõiv et al., 1995) when the latter is colocalized in membrane regions enriched in pyrene-labeled probes and thus yielding decreased excimer fluorescence emission intensity (Lehtonen et al., 1996). In control vesicles the inclusion of DPPF decreased pyrene excimer emission at 480 nm by 40%, whereas in vesicles containing lac permease a 87% decrease in I_E was observed. The latter observation thus reveals DPPF to become enriched into the same microdomains as PPDPC, similar to the observations of DPPG described above.

As I_E/I_M depends on the frequency of collisions between excited and ground-state pyrenes, increasing X_{PPDPC} in POPG yields higher values. To estimate the relative enrichment of PPDPC seen in the presence of lac permease, I_E/I_M was measured as a function of X_{PPDPC} for neat POPG vesicles. To obtain in these vesicles I_E/I_M values similar to those measured at $X_{PPDPC} = 0.005$ in the presence of permease, significantly higher contents ($X_{PPDPC} \approx 0.05$) of the probe were needed (Fig. 4), thus suggesting that a minimally 10-fold enrichment of PPDPC occurs because of the protein.

DISCUSSION

Except for protein-protein contacts, the membrane-spanning segments of integral membrane proteins are surrounded by a shell of adjacent boundary lipids that mediates the coupling between the mostly hydrophobic intramembranous residues of the protein and the lipid bilayer. The secondary structural motifs unambiguously demonstrated so far for integral membrane proteins are the transmembrane α-helix and the β-barrel (Lemon and Engelman, 1994; Haltia and Freire, 1995). Interestingly, transmembrane segments appear to represent the most conserved sequences of membrane-spanning proteins (Kinnunen, 1991). Although this may relate to conserved protein-protein interactions within the membrane, it seems plausible to assume that evolutionally conserved lipid-protein interactions could also be involved.

Acidic phospholipids are essential to the function of a number of integral membrane proteins. It is known that basic amino acids are more prevalent as cytosolic extramembranous residues (von Heijne, 1986), which would be expected to confer a selectivity on the proteins for negatively charged phospholipids. Indeed, acidic phospholipids have been shown to be attracted to the basic residues adjacent to the transmembrane domains of integral membrane proteins (Verbist et al., 1993; Horváth et al., 1995). This notion is further supported by the observation that deletion of a positively charged hydrophilic loop connecting hydrophobic sequences from myelin proteolipid apoprotein causes a large decrease in its preference for acidic phospholipids (Horváth et al., 1990). Another common feature of the sequences residing in the interfacial region is the prevalence of Asn and Gln (Landolt-Marticorena et al., 1993). These residues may ligand because of hydrogen bonding with protonated acidic phospholipids (Rytömaa and Kinnunen, 1995). Aromatic residues Trp, Tyr, and Phe are also abundant in the interfacial region (Landolt-Marticorena et al., 1993), and they could be important in accommodating to the highly dynamic physicochemical heterogeneity of the lipid-water interface (White and Whimley, 1994). In addition to electrostatic interactions, specific lipid-protein interactions have also been reported. Acetylcholine receptor, for instance, needs cholesterol for proper function (Fernandez-Ballester et al., 1994).

An interesting mechanism causing specific lipid-protein interactions relies on the match of the hydrophobic thicknesses of the lipids and transmembrane domains of membrane-spanning proteins (Mouritsen and Sperotto, 1993). Peptides or proteins longer than the membrane thickness increase the acyl chain order of surrounding lipids, whereas shorter proteins and peptides have the opposite effect (Huang, 1986; Huschilt et al., 1985; Riegler and Möhwald, 1986; Kurrle et al., 1990; Zhang et al., 1992; Ludtke et al., 1995; Killian et al., 1996). Hydrophobic mismatch influences both the structure as well as the function of a number of integral membrane proteins. The stability of Band 3, the erythrocyte anion transporter, depends on the hydrophobic thickness of the matrix (Maneri and Low, 1988). Functions of (Ca^{2+}-Mg^{2+})-ATPase, the leucine transport system of *Lactococcus lactis* and rhodopsin, are influenced by hydrophobic thickness (Wiedmann et al., 1988; Gibson and Brown, 1993; Michelangelii et al., 1990; Veld et al., 1991). The activities of Ca^{2+}-ATPase of sarcoplasmic reticulum and cytochrome oxidase are at a maximum with lipids containing 18-carbon acyl chains and decrease substantially with longer and shorter chains (Montecucco et al., 1982). Hydrophobic mismatch may cause protein aggregation as seen for Ca^{2+}-ATPase (Cornea and Thomas, 1994), yet proteins such as Na^{+}-K^{+}-ATPase and bacteriorhodopsin seem to tolerate large thickness variation (Johansson et al., 1981a,b; Lewis and Engelman, 1983). No major effect on the average lipid acyl chain order parameter was observed upon varying the thickness of *Acholeplasma laidlawii* membranes (Thurmond et al., 1994). To this end, we have recently demonstrated that hydrophobic mismatch of constituent lipids may also result in the formation of microdomains in liquid crystalline phospholipid bilayers (Lehtonen et al., 1996). The effective length of PPDPC appears to be best matched by dieicosenoyl PC (i.e., di-20:1 PC) bilayers (Lehtonen et al., 1996).

The present data show that excimer formation by pyrene-labeled phospholipids is strongly enhanced in membranes with reconstituted lactose permease. This increase in excimer formation appears to be due to segregation of the pyrene-labeled phospholipids into the vicinity of this protein. Evidence for the enrichment is provided by the reconstitution studies with the pyrene-labeled mutant protein, which revealed an disproportionally large increase in excimer formation. More specifically, the number of pyrene moieties introduced with the labeled protein is rather small, corresponding to an increase in X_{PPDPC} from 0.005 to 0.00525, whereas a similar value for I_E/I_M to be measured in protein-free liposomes would require $X_{PPDPC} = 0.0055$. Dependency of the observed increase in I_E/I_M on the length of the lipid/protein boundary is demonstrated by the increase in I_E/I_M with increased P/L as well as by the reduction in I_E/I_M due to the aggregation of lac permease by the monoclonal antibody 4B1.

What would be the driving force for the enrichment of the pyrene-labeled lipids in the presence of lactose permease? Specific lipid-protein interactions have not been described for this protein. Based on its primary structure, lac permease has been suggested to have 12 membrane-spanning α-helices, referred to by Roman numerals I–XII and composed of 19 (V), 20 (IX-XII), 21 (VI), 22 (II, IIX), 23 (IV, VII), 24 (I), and 25 (III) amino acids (for a review, see Kaback, 1992; Kaback et al., 1994). Assuming each residue in a membrane-spanning α-helix contributes ~1.5 Å to its length, we may estimate the hydrophobic thicknesses of these α-helical transmembrane domains to range between 28.5 and 37.5 Å. At 25°C the hydrophobic thickness of POPC membrane has been measured to be 25.8 Å (Nezil and Bloom, 1992). Compared to the dimensions measured for crystals of DPPC, for instance, the reason for the thinning of POPC is the considerable extent of *trans→gauche* isomerization of the acyl chains of this phospholipid in the liquid crystalline state. Because of electrostatic repulsion between the acidic headgroups of POPG, the mean molecular areas for this phospholipid can be estimated to be slightly larger than for POPC, and thus the thickness of POPG should also be somewhat less. The bulky and rigid pyrene moiety attached to an acyl chain prevents probes such as PPDPC from adapting to the thickness of POPG by *trans→gauche* isomerization. Accordingly, PPDPC should favor localization to regions where the membrane thickness would better match its effective hydrophobic length.

The increased local membrane thickness due to protein may extend about 10 to 20 surrounding layers of phospholipid into the bilayer (Sperotto and Mouritsen, 1991; Fattal and Ben-Shaul, 1993). Taking into account the above considerations, it seems plausible to assume that proper environment matching the hydrophobic length of PPDPC should surround most of the membrane-spanning helices of permease, and accordingly, enrichment of PPDPC into the vicinity of lactose permease should take place. Accordingly, higher I_E/I_M values are anticipated for vesicles reconstituted with this protein. In further keeping with this notion, the enhancement due to permease of excimer formation by PPHPC, -PG, and -PM is less than for the probes with larger effective lengths, i.e. PPDPC, PPDPG, and PPDPM, the former group of lipids having a six-carbon and the latter a ten-carbon acyl spacer between the pyrene and the glycerol backbone. The repulsive potential for the 6-{(pyrenyl-1)-yl}hexanoyl derivatives PPHPC from POPG, driving them into the vicinity of lac permease due to hydrophobic mismatch, should be less than for the 9-{(pyrenyl-1)-yl}decanoyl lipids. The proposed more efficient enrichment of the fluorescent lipids with longer chains provides strong support for the hydrophobic mismatch being involved. Indeed, the behaviors of the PG, PM, and PC probes were identical, thus suggesting that their enrichment due to lactose permease is not determined by interactions at the lipid headgroup level.

Further evidence for the segregation of PPDPC into microdomains in the presence of permease was provided by experiments in which DPPF was included together with pyrene-labeled lipid in lac permease-containing liposomes. Spectral overlap of pyrene excimer emission and fluores-

cein excitation allows for fluorescence energy transfer between pyrene lipids and DPPF (Kõiv et al., 1995) when the latter is colocalized in membrane regions enriched in pyrene-labeled probes, thus yielding decreased excimer fluorescence. We have recently utilized this property to demonstrate a gradual threefold increase in the excimer formation by PPDPC upon decreasing the acyl chain length of the bulk bilayer phospholipids (Lehtonen et al., 1996). Colocalization of the two probes (DPPF and PPDPC, with hydrophobic parts of similar lengths) was observed when there was a mismatch between the hydrophobic lengths of these lipids and that of the lipid matrix. The mechanism of this colocalization was interpreted to be a hydrophobic mismatch as follows. Incorporation of PPDPC into the thin di-14:1 PC bilayers, for instance, increases the total free energy G of the system. This increase in G arises from the increase in the acyl chain order of the matrix lipids surrounding PPDPC so as to avoid the unfavorable contact of hydrocarbons (0.8 kcal/mol per number of carbons exposed) with water (Tanford, 1973). In the present study the inclusion of DPPF in control vesicles decreased pyrene excimer emission by 40%, whereas in vesicles containing lac permease a 87% decrease in I_E was observed. These data suggest that DPPF colocalizes with PPDPC in the same microdomains in the vicinity of lac permease.

Assuming that the lateral diffusion rates of the probes are not influenced by lac permease comparison of the I_E/I_M values measured at $X_{PPDPC} = 0.005$ in the presence of protein and those for plain liposomes in which X_{PPDPC} was varied from 0.01 to 0.10 reveals that the average effective concentration of PPDPC in the microdomain formed in the presence of permease approximately equals $X = 0.05$. However, this enrichment by a factor of 10 represents an essential minimum when all of the probe should be present in the domains. This is rather unlikely, however, and it is probable that only a fraction of the probes are involved and that the actual enrichment is higher. The probes also appear to move rather freely in these domains, as revealed by the similarity of the I_E/I_M versus temperature data recorded in the absence and presence of lac permease. The factor limiting the extent of the accumulation of a probe such as PPDPC is likely to be their mutual repulsion due to steric elastic strain imposed by the bulky pyrene moiety. This property has previously been assigned to result in the formation of superlattices of this lipid in different fluid phospholipid matrices (Somerharju et al., 1985; Kinnunen et al., 1987).

According to the principle of matching hydrophobic thicknesses (Mouritsen and Bloom, 1984; Sperotto and Mouritsen, 1991), each lac permease residing in a POPG matrix should be surrounded by a lipid domain in which the order of the lipid acyl chains as well as the effective length of the molecules are increased. Our data demonstrate this domain to be further enriched in PPDPC as well as DPPF, i.e., lipids which, because of the length of their acyl chains are repelled from POPG bilayers into the vicinity of lac permease. Inclusion of DPPG ($X = 0.10$) in POPG vesicles decreased the segregation of PPDPC. Compared to the estimated thickness of a POPC bilayer, ~25.8 Å (Nezil and Bloom, 1992), the thickness of a gel-state membrane of DPPC is considerably larger, ~36 Å (Wiener et al., 1989). Sankaram and Thompson (1992) have shown that in binary membranes of DMPC and DSPC, increasing the content of the latter lipid increases the average bilayer thickness. Although quantitative estimates cannot be provided, we can assume that the inclusion of DPPG in POPG increases the average thickness of the membranes. As a result, the driving force for the enrichment of PPDPC in the vicinity of lac permease is attenuated.

As an alternative interpretation of these data, it could be argued that the rigid pyrene moiety would cause the employed fluorescent probes to intercalate between the stiff membrane-spanning α-helical segments of lac permease. However, it is difficult to reconcile how this should enhance the rate of excimer formation. To this end, it could also be postulated that there is a binding site in lac permease accommodating two PPDPC molecules so as to enhance excimer formation. Yet this would require such a site to be relative specific for PPDPC, preferring this fluorescent analog over PPHPC. The above mechanisms would additionally require a binding site for DPPF to be present in lac permease as well. Furthermore, a binding site for PPDPC in the protein is not consistent with the decrease in I_E/I_M observed due to the incorporation of DPPG in the liposomes. Last, the thermal augmentation of the increase in I_E/I_M in both the presence and absence of lac permease is similar, again speaking against a PPDPC dimer-accommodating pocket in this protein as an explanation of the observed increase in I_E/I_M. To this end, our preliminary results with porin ompF, another membrane-spanning protein (Engel et al., 1985; Weiss et al., 1991; Cowan et al., 1992), revealed this protein not to cause microdomain formation by the pyrene-labeled lipids (Lehtonen and Kinnunen, unpublished results). The nonpolar surface of porin from *Rhodobacter capsulatus* has a thickness of 25 Å, i.e., very close to that of POPG (Weiss et al., 1991).

Biomembranes are best understood as a strongly correlated two-dimensional "fluids" with a high rate of lateral diffusion concomitant with lateral organization (Mouritsen and Kinnunen, 1996). Under physiological conditions most biological membranes are above their main phase transition temperature, thus emphasizing the importance of understanding the molecular mechanisms generating compositional fluctuations and microdomains in fluid bilayers. Complete mixing of the membrane constituents in a system is caused by entropy-driven randomization. In membranes containing proteins and lipids with large variation in hydrophobic thickness, the minimum energy state of the system is represented by enrichment of proteins and lipids with matching hydrophobic thickness into microdomains, so as to minimize exposure of their hydrophobic moieties to water (Sperotto and Mouritsen, 1991). Although the actual size (or, more accurately, the coherence length of the pyrene-lipid-enriched domains) cannot be estimated from our data, the present study clearly provides experimental

evidence for the validity of hydrophobic matching conditions. Finally, it must also be emphasized that the domains formed by matching of the thicknesses of the hydrophobic domains of lac permease and lipids are not static, but are highly dynamic structures, although the lateral mobility of lipids may be reduced compared to the bulk lipid.

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