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Lipid enrichment and selectivity of integral membrane proteins in two-component lipid bilayers

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Abstract. A model recently used to study lipid-protein interactions in one-component lipid bilayers (Sperotto and Mouritsen, 1991 a, b) has been extended in order to include two different lipid species characterized by different acyl-chain lengths. The model, which is a statistical mechanical lattice model, assumes that hydrophobic matching between lipid-bilayer hydrophobic thickness and hydrophobic length of the integral protein is an important aspect of the interactions. By means of Monte Carlo simulation techniques, the lateral distribution of the two lipid species near the hydrophobic protein-lipid interface in the fluid phase of the bilayer has been derived. The results indicate that there is a very structured and heterogeneous distribution of the two lipid species near the protein and that the protein-lipid interface is enriched in one of the lipid species. Out of equilibrium, the concentration profiles of the two lipid species away from the protein interface are found to develop a long-range oscillatory behavior. Such dynamic membrane heterogeneity may be of relevance for determining the physical factors involved in lipid specificity of protein function.

Key words: Lipid bilayer – Binary mixture – Lipid-protein interaction – Lipid selectivity

I. Introduction

One of the unresolved problems in modern membrane biology is related to the question of whether the activity of membrane-bound proteins and enzymes is specific with respect to certain types of lipids and whether such specificity is controlled by chemical or physical factors (Devaux and Seignoret 1985). Related to this problem is the possible functional significance of membrane domains (Edidin 1990; Mouritsen and Jørgensen 1992; Thompson et al. 1992; Mouritsen and Biltonen 1993) characterized by a static or a dynamic inhomogeneous lateral distribution of membrane molecular components. The biological membrane is a complex many-particle system with significant dynamics (Bloom et al. 1991). Owing to the mutual physical and chemical interactions among the membrane constituents and owing to the fluxes of various forms of energy, the functional membrane organizes itself into a non-equilibrium steady-state. The molecular structure of the membrane in this steady-state is a highly non-trivial consequence of membrane cooperativity and it reflects in an intimate way the nature of the various interactions, in particular the lipid-protein interactions. Via the lipid-protein interactions, the membrane-bound proteins on the one side control the lateral distribution of the lipid molecules, and the lipids and their structure and dynamics on the other side influence protein structure and function and hence couple to the overall membrane physiology.

In order to penetrate the very complex behavior of a many-particle membrane system and its cooperative behavior it is important, as a first step, to come to grips with the fundamental and basic consequences of simple and well-defined lipid-protein interactions in membranes and to which extent particular physical forces may be responsible for membrane organization and lateral heterogeneity. To this end we consider in the present paper a simple microscopic model of lipid-protein interactions with a view to determining to which extent bare physical effects may be relevant for lipid selectivity and specificity of membrane proteins. The basic idea behind the model is that, via a hydrophobic matching condition, the lipid chains of varying length feel the perturbation of the protein hydrophobic surface to different extents. It is therefore anticipated that the lipid species which can most easily adapt to the matching condition will be selected by the protein, on a statistical basis, and will have an increased probability of being close to the lipid-protein interface. This effect is an example of interface enrichment. In our model a number of other forces, e.g. electrostatic interactions, which may be relevant in certain lipid-protein systems have been neglected in order to isolate the effects due to hydrophobic mismatch.

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Lipid enrichment phenomena are likely to contribute to the functional specialization of membrane regions (Sackmann 1984; Kinnunen 1991). The structure and dynamics of the lipid bilayer matrix is known to control the activity of a number of different enzymes. For example, the enzymatic activity of (Na^+-K^+)-ATPase (Johansson et al. 1981) and Ca^{2+}-ATPase (Caffrey and Feigenson 1981) is maximal when reconstituted into lipid bilayers of a given thickness (Mouritsen and Sperotto 1992). The enrichment of a region around the protein in a particular type of lipid species may be a way to modulate the enzymatic activity of proteins, and the effect of the enrichment on membrane-active compounds, such as drugs, may provide a clue to the mechanism of drug action.

II. Microscopic model

The model used in the present paper is an extension of a microscopic model (Sperotto and Mouritsen 1991 a, b) recently used to describe a one-component lipid bilayer incorporated with a dilute dispersion of proteins with particular emphasis on lipid-order parameter profiles around static integral membrane proteins and the aggregational state of small mobile integral proteins. This model is now extended in order to include two different lipid species characterized by two different acyl-chain lengths. The model is built on the ten-state Pink model (Pink et al. 1980) of the main phase transition of one-component lipid bilayers (Mouritsen 1991). The Pink model accurately accounts for the most important conformational states of the lipid chains and their mutual interactions and statistics. This model has proved useful to describe a wealth of thermodynamic, thermomechanic, and spectroscopic data for a variety of phospholipid membranes (Mouritsen 1990, 1991). Within the Pink model, the bilayer is formed by two independent monolayers, each represented by a triangular lattice, on which the lipid chains are arrayed, one chain at a site. The two monolayers of the bilayer are hence assumed to be identical and possible effects on the lipid-protein interactions due to lipid asymmetry are neglected. Each lipid acyl chain on the lattice can take on one of ten conformational states, m, each of which is characterized by a hydrocarbon chain length, d_m. Within the lattice formulation, a protein molecule is taken to occupy one or more lattice sites and its hydrophobic part is assumed to be smooth, rod-like, and characterized only by a cross-sectional area, A_P, a circumference ϱ_P, and a half-length of the hydrophobic protein domain, d_P. In the following, the term 'protein length' will refer to d_P.

The lipid-protein interactions have been incorporated into the microscopic Pink model by identifying part of the interaction parameters in terms of hydrophobic matching between the hydrophobic length of the lipid chain and hydrophobic protein length in the spirit of the phenomenological mattress model of lipid-protein interactions (Mouritsen and Bloom 1984, 1993). The interaction between lipids of the two different species α, $\alpha=1, 2$, is formulated in terms of an attractive van der Waals-like interaction and a repulsive 'mismatch' interaction which accounts for the possible incompatibility between the chain lengths of the two different lipid species.

The total Hamiltonian for the model is then written

$$\mathcal{H}=\sum_{\alpha}\left(\mathcal{H}_{L}^{0,\alpha}+\mathcal{H}_{L-P}^{\alpha}+\frac{1}{2}\sum_{\alpha'\neq\alpha}\mathcal{H}_{L-L}^{\alpha,\alpha'}\right), \tag{1}$$

where

$$\begin{aligned}\mathcal{H}_{L-P}^{\alpha}=\Pi\, A_P\sum_i L_{P,i}&\\ +\frac{\gamma_{mis}}{2}\left(\frac{\varrho_P}{Z}\right)\sum_{\langle i,j\rangle}\sum_m&(|d_{m,i}^{\alpha}-d_P|\,\mathcal{L}_{m,i}^{\alpha}L_{P,j}\\ &+|d_{m,j}^{\alpha}-d_P|\,\mathcal{L}_{m,j}^{\alpha}L_{P,i})\\ -\frac{v_{vdW}}{2}\left(\frac{\varrho_P}{Z}\right)\sum_{\langle i,j\rangle}\sum_m&(\min(d_{m,i}^{\alpha},d_P)\,\mathcal{L}_{m,i}^{\alpha}L_{P,j}\\ &+\min(d_{m,j}^{\alpha},d_P)\,\mathcal{L}_{m,j}^{\alpha}L_{P,i})\end{aligned} \tag{2}$$

$$\begin{aligned}\mathcal{H}_{L-L}^{\alpha,\alpha'}=-\frac{J}{2}\sum_{\langle i,j\rangle}\sum_{m,n}&(f(d_{m,i}^{\alpha},d_{n,j}^{\alpha'})\,\mathcal{L}_{m,i}^{\alpha}\mathcal{L}_{n,j}^{\alpha'}\\ &+f(d_{m,i}^{\alpha'},d_{n,j}^{\alpha})\,\mathcal{L}_{m,i}^{\alpha'}\mathcal{L}_{n,j}^{\alpha})\\ +\frac{\Gamma}{2}\sum_{\langle i,j\rangle}\sum_{m,n}&(|d_{i,m}^{\alpha}-d_{j,n}^{\alpha'}|\,\mathcal{L}_{m,i}^{\alpha}\mathcal{L}_{n,j}^{\alpha'}\\ &+|d_{i,m}^{\alpha'}-d_{j,n}^{\alpha}|\,\mathcal{L}_{m,i}^{\alpha'}\mathcal{L}_{n,j}^{\alpha}).\end{aligned} \tag{3}$$

$\mathcal{H}_L^{0,\alpha}$ is the Pink Hamiltonian for the pure lipid bilayer of species α. $\mathcal{H}_{L-P}^{\alpha}$ is the lipid-protein Hamiltonian as described in detail elsewhere (Sperotto and Mouritsen 1991 a, b). The parameter v_{vdW} is related to the direct lipid-protein van der Waals-like interaction which is associated with the interfacial hydrophobic contact of the two molecules, while the parameter γ_{mis} is related to the hydrophobic effect. $\mathcal{H}_{L-L}^{\alpha,\alpha'}$ is the lipid-lipid Hamiltonian for interacting chains associated with different lipid species. J is the strength of the van der Waals interaction between neighboring chains taken to be $J=\sqrt{\prod_\alpha J_0^\alpha}$, where J_0^α is the Pink model interaction parameter for the pure lipid bilayer (Pink et al. 1980), and Γ is the 'mismatch' interaction parameter. $f(d_{m,i}^{\alpha},d_{n,j}^{\alpha'})$ is an interaction matrix which involves both distance and shape dependence. $\mathcal{L}_{m,i}^{\alpha}=0,1$ ($\alpha=1,2$) are the occupation variables for the two lipid species and $L_{P,i}=0,1$ is the protein occupation variable. $\mathcal{L}_{m,i}^{\alpha}$ and $L_{P,i}$ satisfy a completeness relation at each lattice site, $\sum_\alpha\left(\sum_m\mathcal{L}_{m,i}^{\alpha}\right)+L_{P,i}=1$.

The values of the lipid-protein interaction parameters, γ_{mis} and v_{vdW}, have been chosen in accordance with those of the phenomenological mattress model (Sperotto and Mouritsen 1988): $\gamma_{mis}=0.01\times10^{-13}$ erg Å^{-2} and $v_{vdW}=0.33\times10^{-13}$ erg Å^{-2}. The 'mismatch' lipid-lipid interaction parameter Γ has been chosen (Jørgensen et al. 1993) in order to reproduce the strong non-ideal phase behavior found experimentally for binary lipid mixtures with acyl-chains length differences of at least four carbon atoms (Mabrey and Sturtevant 1976): $\Gamma=0.038\times10^{-13}$ erg Å^{-1}. The accordance between the experimental phase diagram for the binary DMPC-DSPC system[1] and the diagram, which is obtained for the mi-

[1] DMPC: dimyristoyl phosphaticylcholine; DSPC distearoyl phosphaticylcholine

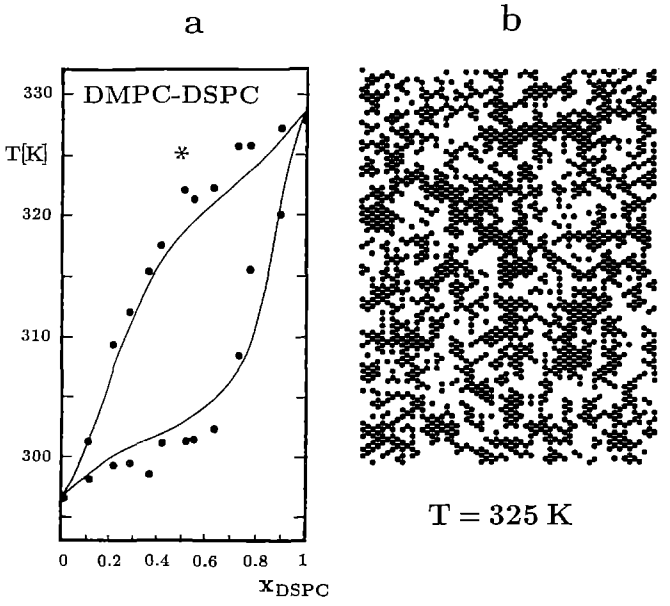

Fig. 1. **a** Phase diagram for the DMPC-DSPC lipid mixture. Data are shown as obtained from differential scanning calorimetry (●) and from theoretical calculations (*solid line*) based on the model in (1)–(3). **b** Snapshot of a microconfiguration typical of equilibrium of a DMPC-DSPC mixture at $T=325$ K and $x_{DSPC}=0.5$ as calculated by computer simulation of the model in (1)–(3). The system contains 60×60 lipid chains. Black regions correspond to DMPC and white regions to DSPC

croscopic model described in (1)–(3) with the parameter values quoted above, is shown in Fig. 1 a.

III. Calculational method

In the present work we have studied the limit of a very large protein, i.e. a protein whose cross-sectional diameter is much larger than the cross-sectional diameter of a lipid-acyl chain. The protein is assumed to be immobile in the lipid matrix. This limit is interesting since it will show the maximal effect of lipid-protein interface enrichment. The limit will be relevant for large proteins, such as the photosynthetic reaction center of *rhodopseudomonas viridis* (Deisenhofer et al. 1985) or bacteriorhodopsin from *halobacterium halobium* (Henderson and Unwin 1975). The hydrophobic surface of the large protein is modelled by a linear boundary condition on the lipid-bilayer lattice. Since this lattice is subject to periodic boundary conditions, the model system in fact includes two lipid-protein interfaces, and the structure of the binary lipid-membrane matrix will be symmetric about the middle line of the lattice.

The microscopic and the macroscopic thermodynamic properties of the present model of lipid-protein interactions in binary lipid mixtures in equilibrium have been calculated by Monte Carlo computer-simulation techniques (Mouritsen 1990). By these numerical techniques it is possible to derive the thermodynamic properties from the model Hamiltonian without invoking any approximations. Hence all of the model properties are basically derived from first principles. The simulations are performed in the canonical ensemble specified by the temperature and the composition. In this ensemble, the system is coupled to a heat bath which provides the energy needed for bringing about the equilibrium state and its thermal statistics. Since the simulations provide the microstates of the entire system, it is furthermore possible from these calculations to determine the molecular organization of the membrane components. Finally, it is possible, via the Master-equation interpretation of the Monte Carlo process to obtain dynamic information on diffusional processes in the membrane plane (Mouritsen 1990). We have in the present work calculated the specific heat, $C_p(T)$, and the normalized lipid concentration profiles, $P(r)$ as a function of the distance, r, from the surface of the protein. Owing to symmetry, $P(r)$ is calculated as an average over the two halves of the lattice.

The Monte Carlo simulations are carried out on a triangular lattice of 60×60 sites. On this lattice, the protein has a hydrophobic surface extending over 60 sites. The thermal equilibrium is provided for by a combination of single-chain conformational excitations and diffusive exchange of nearest neighbor chains.

The results discussed below refer to the case of mixtures with DMPC and DSPC phospholipids. The results for other binary mixtures of saturated diacyl phosphatidylcholines are qualitatively similar. However, the larger the difference in acyl-chain length, the more pronounced are the enrichment effects.

IV. Results and discussion

We first describe the results for a binary DMPC-DSPC mixture in the absence of proteins in order to determine the phase behavior of the mixture and in order to assess the lateral structure in the mixed fluid phase as it is controlled by thermal density fluctuations alone. Binary mixtures of DMPC and DSPC exhibit a strongly non-ideal phase behavior. The mixture exhibits a pronounced gel-fluid lateral phase separation in the temperature range between the transition temperatures of the two lipid components (Mabrey and Sturtevant 1976) as shown in the phase diagram in Fig. 1 a. This phase diagram is calculated by mean-field theory. In the phase-separation region, the membrane is laterally heterogeneous in a static fashion due to plain phase coexistence. In the fluid one-phase region above the upper phase boundary in Fig. 1 a the binary membrane is, however, also heterogeneous, although in a dynamic fashion. Owing to the thermal fluctuations prevailing in the neighborhood of the transition (Mouritsen 1991), the bilayer has a local structure on a mesoscopic length scale corresponding to the coherence length of the lateral fluctuations. The resulting dynamic heterogeneity (Mouritsen and Jørgensen 1992; Jørgensen et al. 1993) is illustrated in Fig. 1 b which gives a snapshot of a membrane configuration typical of equilibrium close to and above the phase boundary. The configuration is calculated by Monte Carlo simulation of the microscopic model of the binary lipid mixture. It is this dynamic heterogeneity and its typical coherence length which is

picked up by the lipid-protein interactions and which leads to lipid enrichment and physical selectivity of integral membrane proteins.

We now consider a system with a static protein embedded in an equimolar mixture of DMPC and DSPC. Figure 2 shows the simulation results for the lipid concentration profiles, $P(r)$, for the two lipid species as a function of the distance, r, from the lipid-protein interface. Two situations are considered: (*a*) the hydrophobic half thickness of the protein, $d_P = 13$ Å, is chosen to be close to the value of the typical acyl-chain length of DSPC molecules in the fluid phase, and (*b*) the hydrophobic half thickness of the protein, $d_P = 10$ Å, is chosen to be close to the value of the typical acyl-chain length of DMPC molecules in the fluid phase. The simulations are performed at the temperature $T = 325$ K which is close to and above the phase boundary, cf. Fig. 1. Figure 2 shows that the protein selects the lipid species, DSPC in case (*a*) and DMPC in case (*b*), which most easily wets its hydrophobic surface. The lipid-protein hydrophobic interface is consequently enriched in one species and simultaneously depleted in the other one. This selectivity is a direct consequence of the hydrophobic matching condition. The lipids are selected on a statistical basis, and their lateral distribution close to the protein is not static but dynamic.

The profiles shown in Fig. 2 demonstrate that the range over which the protein influences the lateral distribution of the lipids may be quite extended and involve several lipid layers around the protein. In the neighborhood of the protein, the lipid composition is hence very different from that in the bulk. The decay to the bulk value of the composition may effectively be described by two exponentials separated by a crossover around 3–4 lattice spacings. The decay constant (coherence length) is larger at longer distances. For a fixed composition in the fluid phase, the coherence length increases as the temperature is lowered towards the phase boundary. The exponential decay of the profile can be explained by a simple Landau-de Gennes-type theory (Owicki et al. 1978; Binder 1983). From a comparison of the two cases in Fig. 2 it is seen that the enrichment near the protein of DSPC in case (*a*) is more pronounced than the enrichment of DMPC in case (*b*). This result can be qualitatively understood if one compares, for the chosen temperature, the interaction energies between lipids and protein in the two cases. Although the lipid-protein repulsive interaction energies are roughly the same for DMPC in case (*b*) as for DSPC in case (*a*), the van der Waals lipid-protein attractive interaction energy, which is proportional to the total hydrophobic contact, will favor the larger lipid-protein interfacial contact, i.e. DSPC in contact with the longer protein.

It is noted from Fig. 2 that the lipid profiles do not decay to the value 0.5 expected for a equimolar mixture. This is a finite-concentration effect. Only for a system with an infinite protein dilution (i.e. for an infinite lattice in the present implementation) does $P(r)$ decay to the bulk composition. This is a very important, although trivial, observation since it implies that the profiles of the two proteins in the present set-up overlap, leading to an effective lipid-mediated protein-protein attraction which would have consequences for a membrane system with mobile proteins.

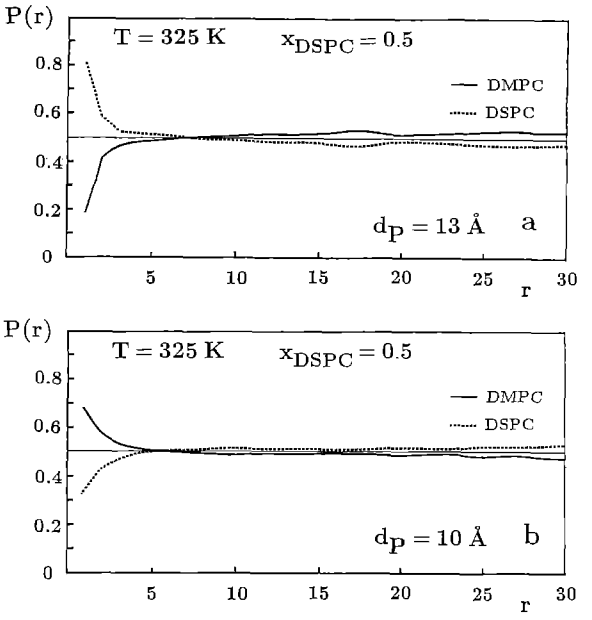

Fig. 2a, b. Lipid concentration profiles, $P(r)$, for an equimolar mixture of DMPC and DSPC, as a function of the distance, r, from a very large integral membrane protein of hydrophobic length $d_P = 13$ Å **a** and $d_P = 10$ Å **b**. The data are obtained from computer simulations on a system with 60×60 lipid chains and refer to a temperature of $T = 325$ K

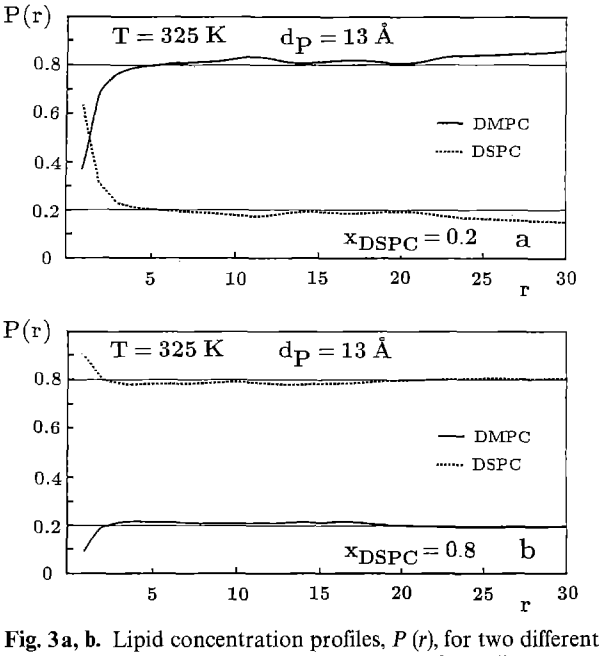

Fig. 3a, b. Lipid concentration profiles, $P(r)$, for two different mixtures of DMPC and DSPC, as a function of the distance, r, from a very large integral membrane protein of hydrophobic length $d_P = 13$ Å. **a** $x_{DSPC} = 0.2$ and **b** $x_{DSPC} = 0.8$. The data are obtained from computer simulations on a system with 60×60 lipid chains and refer to a temperature of $T = 325$ K

In Fig. 3 are shown the results of the concentration profiles for two other compositions, $x_{DSPC} = 0.2$ and 0.8, of the binary DMPC-DSPC mixture in the case of a protein hydrophobic thickness of $d_P = 13$ Å at a temperature of

$T = 325$ K. By comparison with the results for the equimolar mixture in Fig. 2, it is seen that the same qualitative picture applies involving an enrichment of one of the lipid species at the protein-lipid interface and a concomitant depletion of the other species. Both of the mixtures in Fig. 3 are in the fluid phase, cf. Fig. 1 a.

The findings reported above show that the lipid-protein interactions may induce a considerable degree of compositional heterogeneity in the binary lipid mixture. In contrast it is found that for most temperatures in the fluid phase, the acyl-chain orientational order parameter is only affected in the lipid layer in contact with the protein. This is due to the fact that the fluctuations in the acyl-chain order are thermally controlled, and only when close to a phase boundary is the lipid-order parameter profile found to extend beyond the first layer (Sperotto and Mouritsen 1991 b). The possibility of a very extended lipid order-parameter profile around a large integral membrane protein was demonstrated experimentally by Rehorek et al. (1985) and most recently by Piknova et al. (1993) in the case of bacteriorhodopsin.

A striking observation made from our model simulations is related to a non-equilibrium transient effect found in the different concentration profiles of the two lipid species as these profiles establish themselves in the course of time. This effect, which we believe may have some important consequences for steady-state membrane organization, refers to a situation where a thermally equilibrated binary lipid mixture is prepared in the fluid phase and is then suddenly subjected to a change in the boundary condition imposed by the presence of the proteins. The sudden change in boundary conditions could, for example, be induced by conformational changes in the protein and should not be considered as a consequence of the process of insertion of the protein into the membrane. In response to the presence of the proteins, the mixture has to reorganize itself laterally and decompose locally as illustrated by the concentration profiles in Figs. 2 and 3. This reorganization proceeds via long-range diffusional processes. The interdiffusion of the species is, however, limited by the conservation law imposed by the global composition of the mixture. Figure 4 shows that the mixture, on its way to equilibrium in the presence of proteins, displays a pronounced oscillatory behavior in the concentration profile. This behavior is dictated by the diffusional processes and the mass-conservation law: after introduction of the proteins to the initially equilibrated mixture, the protein surfaces are, on a time scale corresponding to short-range diffusion, enriched in the appropriate species whose hydrophobic acyl-chain length is compatible with the protein thickness. However, on this time scale the mixture does not have time to fully reorganize and compensate for the excess mass of the enriched species. The reorganization only occurs on a much longer time scale corresponding to long-range diffusion. This leads therefore to a depletion layer next to the enrichment layer in the same species which is enriched near the protein. Since the other species have to follow suit by the opposite series of local depletion and enrichment layers, a full oscillatory behavior develops as seen in Fig. 4. As time elapses, the nodes of the

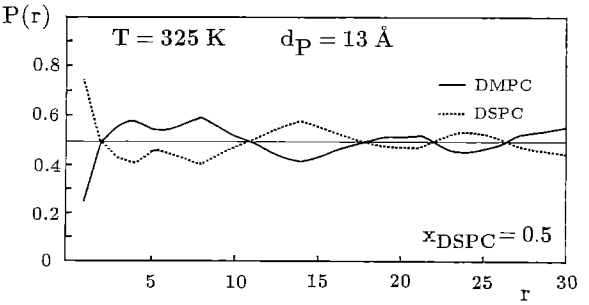

Fig. 4. Transient oscillatory behavior of the lipid concentration profiles, $P(r)$, for an equimolar mixture of DMPC and DSPC, as a function of the distance, r, from a very large integral membrane protein of hydrophobic length $d_P = 13$ Å. The data are obtained from computer simulations on a system with 60×60 lipid chains and refer to a temperature of $T = 325$ K

oscillations move towards larger values of r and eventually dampen out and the equilibrium concentration profiles in Fig. 2 are recovered. Since our results are derived from Monte Carlo simulations which build on stochastic dynamics we are unable to estimate the time duration of the oscillatory behavior.

The results presented above refer to the case of immobile model proteins, such as proteins bound to specific positions of the membrane, for example via the cytoskeleton, or to proteins which diffuse very slowly relative to the lipids. However, in the case of mobile proteins we can anticipate from the general nature of our results for static proteins that the structured concentration profiles, cf. Figs. 2 and 3, will facilitate a medium-range lipid-mediated indirect protein-protein attraction which will have influence on the state of protein aggregation. This observation may have biological relevance for those proteins whose biological activity depends on their aggregational state (Kaprelyants 1988; Andersen 1989).

It is interesting to note that for a non-equilibrium system, say a protein-lipid membrane driven by external sources of energy, e.g. chemical reactions or radiation fields, which couple to protein conformational changes, the oscillatory profile in Fig. 4 may be dynamically maintained. The mobile proteins may in the driven system organize themselves laterally to fit into the part of the profile which is enriched in the lipid species with the higher affinity for the protein. This picture may be directly generalized to systems of proteins with different lipid selectivity.

The selectivity phenomenon described above belongs to a more general class of surface-enrichment phenomena encountered in a variety of physical systems (Binder 1983) including binary alloys and polymer mixtures. Under the influence of an external perturbation, like a wall which favors one of the two components of the mixture, these systems show an enrichment in this component near the wall. The transient oscillatory behavior in the concentration profile found in the present work for lipid-protein systems, cf. Fig. 4, is related to a similar recent finding in simple binary fluid and solid mixtures (Binder and Frisch 1991). It is also interesting to point out the possibility that there may be parts of the phase diagram in which the enrichment equilibrium profiles in Figs. 2 and 3 may de-

velop into a complete wetting phenomenon, which implies that the enriched layer becomes macroscopically large (Dietrich 1988). Wetting phenomena would have a pronounced effect on the heterogeneous membrane structure.

Recent studies have suggested that foreign molecules, like drugs, tend to accumulate along lines of membrane defects (Jørgensen et al. 1991). By 'lines of membrane defects' one generally means the dynamic interfaces between the fluid and the gel domains which are formed in the transition region and which are induced by thermal fluctuations. It would be of interest to investigate if this accumulation also occurs in the 'compositional' interfaces implied by the enrichment and depletion layers in the lipid-protein system. Such an accumulation would increase the probability of forming direct contacts between the proteins and drugs. The formation of such contacts could be important for the functional properties of some integral proteins. Specifically, a number of studies indicate that general anesthetics, besides acting non-specifically via the lipid matrix (Miller et al. 1989), also appear to bind specifically to proteins and receptors, altering their activity. This seems to be the case for example for neuronal Ca^{2+}-channels (Kress et al. 1991) which are involved in the presynaptic release of neurotransmitters.

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