A Monte Carlo Simulation Study of Protein-Induced Heat Capacity Changes and Lipid-Induced Protein Clustering

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ABSTRACT Monte Carlo simulations were used to describe the interaction of peripheral and integral proteins with lipids in terms of heat capacity profiles and protein distribution. The simulations were based on a two-state model for the lipid, representing the lipid state as being either gel or fluid. The interaction between neighboring lipids has been taken into account through an unlike nearest neighbor free energy term Δω, which is a measure of the cooperativity of the lipid transition. Lipid/protein interaction was considered using the experimental observation that the transition midpoints of lipid membranes are shifted upon protein binding, a thermodynamic consequence of different binding constants of protein with fluid or gel lipids. The difference of the binding free energies was used as an additional parameter to describe lipid-protein interaction. The heat capacity profiles of lipid/protein complexes could be well described for both peripheral and integral proteins. Binding of proteins results in a shift and an asymmetric broadening of the melting profile. The model results in a coexistence of gel and fluid lipid domains in the proximity of the thermotropic transition. As a consequence, bound peripheral proteins aggregate in the temperature range of the lipid transition. Integral proteins induce calorimetric melting curves that are qualitatively different from that of peripheral proteins and aggregate in either gel or liquid crystalline lipid phase. The results presented here are in good agreement with calorimetric experiments on lipid-protein complexes and have implementations for the functional control of proteins.

INTRODUCTION

Biomembranes are dynamic systems with many components, consisting mainly of lipids and proteins. These are all flexible molecules with numerous possible arrangements and conformations. Thus, physical properties of a membrane are the properties of a macroscopic ensemble rather than the sum of the properties of the individual components. The physical behavior of lipids in a membrane can couple to the behavior of proteins through a variety of interactions.

A few cases are known in which the function of lipid/protein complexes are correlated with the physical state of the lipid membrane. These include cytochrome c (Heimburg et al., 1991; Heimburg and Marsh, 1993), α-β-hydroxybutyrate dehydrogenase (Cortese and Fleischer 1987), gramicidin A (Huang, 1986; Elliott et al., 1983; Cornell et al., 1989; O’Connell et al., 1990), the alamethicin pore (Boheim et al., 1980; Vodyanoy et al., 1993; Keller et al., 1994), phospholipase A2 (Romero et al., 1987; Bell and Biltonen, 1989; Biltonen, 1990); protein kinase C (PKC; Nishizuka, 1984, 1986; Orr and Newton, 1992a,b). In most or all cases where it has been investigated, the thermotropic melting reaction of the lipid membrane is affected by the presence of peripheral or integral proteins. Usually this results in a shift in the heat capacity maximum and a broadening of the melting profile. In some cases the transition enthalpy is also reduced. Examples are the binding of cytochrome c (Heimburg and Biltonen, 1994; deKruijff and Cullis, 1980) and myelin basic protein (Ramsay et al., 1986) to charged lipid membranes or the interaction of the integral band 3 protein of erythrocytes (Morrow et al., 1986), the transferrin receptor (Kurle et al., 1990), and cytochrome b5 with neutral lipids (Freire et al., 1983). In some membranes, proteins appear to form aggregates as a consequence of lipid transitions or changes in lipid composition (Mouritsen and Sperotto, 1992), e.g., in membranes containing bacteriorhodopsin (Lewis and Engelman, 1983) or rhodopsin (Mouritsen and Sperotto, 1992). Band 3 protein aggregation within lipid domains in erythrocyte membranes has been reported (Rodgers and Glaser, 1993). For the peripheral phospholipase, A2 aggregation equilibria have been discussed as being relevant for the enzymatic activity (Bell and Biltonen, 1989; Biltonen, 1990). Gramicidin A and the peptide alamethicin form channels or pores that consist of dimers or aggregates (Gennis, 1989). These aggregation equilibria in membranes might well be influenced by the lipid state. The lipid state thus can be viewed as a possible basis for the general control of protein function in biological membranes through its effects on protein association or clustering.

In some earlier works, thermotropic lipid melting was modeled assuming only two states of the lipid, gel and fluid, using an interfacial free energy term between adjacent lipids to rationalize the cooperativity of the transition (Doniach,
1978; Sugar et al., 1992, 1994; Mouritsen and Bilston, 1992). This approach was first used by Zimm and Bragg (1959) to explain the cooperative unfolding of one-dimensional polyamide helices. Marsh et al. (1977) originally attempted to extend this concept to lipid membranes. In this model the membrane is not considered as static but as a dynamic ensemble of lipid molecules. All physical observables are the time average over all possible states of the system, and no analytical procedure of determining the number and nature of states is known. Therefore, one uses Monte Carlo simulations to extract the population of each state of a membrane. The statistical fluctuations in energy are related to the heat capacity function describing the membrane transition (fluctuation dissipation theorem; Hill, 1960). The existence of finite interfacial energies leads to the coexistence of gel/fluid lipid domains in the phase coexistence region. The putative significance of such domains has been discussed extensively as being an important factor in the regulation of membrane function (Mouritsen and Bilston, 1992; Fraser et al., 1991; Mouritsen, 1989, 1991; Mouritsen et al., 1992; Thompson et al., 1992, 1995).

Monte Carlo simulations have been used to rationalize the shape of the calorimetric melting profile including a 10-state model for the transition, first proposed by Pink and Chapman (1979) and Pink et al. (1980). In this more complex approach one considers van der Waals interactions as well as membrane lateral pressure and an interfacial energy. This description therefore requires a number of parameters and makes some important predictions on the molecular level (Mouritsen and Sperotto, 1992; Mouritsen and Bilston, 1992; Fattal and Ben-Shaul, 1993; Sperotto and Mouritsen, 1991, 1993; Sperotto et al., 1989). However, the physics that leads to domain formation and that is necessary to rationalize the heat capacity profiles is already contained in the much simpler two-state Ising model with only an additional interfacial energy term. This interfacial energy term is the only parameter required for the calculation that is not determined by experiment. In this paper we use a two-state Ising model to describe the heat capacity profiles and the lateral distribution of lipids in the presence of peripheral and integral proteins. In addition, these simulations demonstrate how changes in lipid structure/composition can lead to changes in the local clustering of protein on and within the membrane.

**THE MODEL**

*Ising model for the gel-fluid transition*

The lipid transition shall be described assuming that only two states, gel and fluid, are available for each individual lipid. These lipids are situated in a planar matrix with a triangular lattice (six nearest neighbors/site) and sites. Lipid-lipid interactions are taken into account through a nearest neighbor interaction free energy $\varepsilon$. The free energy of each individual lipid molecule consists of two components, which are the intrinsic free energy $G_i$ (the index $i$ stands for $g$ (gel) or $l$ (fluid)) of the chain configuration and the sum over the nearest neighbor interaction free energies $\varepsilon_{ij}$. As long as the transition half-width is small, it is a reasonable simplification to assume that the nearest neighbor interactions $\varepsilon_{ij}$ are purely enthalpic. The total free energy of the lipid matrix in the absence of protein is the sum over all $n$ lipids:

$$G = n_g \cdot G_g + n_l \cdot G_l + n_{gg} \cdot \varepsilon_{gg} - n_l \cdot \varepsilon_{gl} - n_g \cdot \varepsilon_{lg},$$

where $n_i$ is the number of lipids in state $i$, and $n_{ij}$ is the number of nearest neighbor interactions of lipids in states $i$ and $j$. This can be rewritten as

$$G = n \cdot G_g + n \cdot (\Delta H - T \cdot \Delta S) - n_{gl} \cdot \Delta \varepsilon_{gl}.$$

Defining $\Delta H = (H_g - z \cdot \varepsilon_{gg}) - (H_l - z \cdot \varepsilon_{lg})$ and $\Delta S = (S_g - S_l)$, with $z = 6$.

The excess free energy of the system is given by

$$\Delta G_{ex} = G - n \cdot G_g = n_l \cdot (\Delta H - T \cdot \Delta S) - n_l \cdot \Delta \varepsilon_{gl}.$$

Rearranging, the cooperative nature of the transition is given by

$$\Delta G_{ex} = \le 0,$$

The magnitude of $\Delta \varepsilon_{gl} = \varepsilon_{gg} - \varepsilon_{lg}$ defines the transition. If $\Delta \varepsilon_{gl} = 0$, the transition is non-cooperative: each lipid melts independently, producing a very broad transition. In this case the heat capacity function

$$C_p(T) = \frac{e^{-\Delta G/T}}{1 + e^{-\Delta G/T}} \cdot \frac{\Delta H^2}{RT^2}.$$

If $\Delta \varepsilon_{gl} = \infty$, the melting is an all-or-none transition; each vesicle of size $N$ is either completely gel or completely fluid and the transition is very sharp. In this case,

$$C_p(T) = \frac{e^{-\Delta G/T}}{1 + e^{-\Delta G/T}} \cdot \frac{N \cdot \Delta H^2}{RT^2}.$$

However, the melting profile will be of finite width because $N$ is finite. As $N \rightarrow \infty$, classical first-order behavior will be observed.

At the transition temperature, $T_m$, $\Delta G = \Delta H - T_m \cdot \Delta S$ is equal to 0 and $\Delta S = \Delta H / T_m$. The enthalpy $\Delta H$ and $T_m$ can

**MATERIALS AND METHODS**

Cytochrome c, oxidized form type VI, Sigma Chemical Co., St. Louis, MO, was used without further purification. Dmyristoylphosphatidylglycerol (DMG), Avanti Polar Lipids, Birmingham, AL, was checked by thin-layer chromatography and used without further purification. 2 mM Hepes and 1 mM EDTA (pH 7.5) were used as buffers. At low ionic strength pure DMG exhibits a highly cooperative transition only at high concentrations (>150 mM_Heinemann and Bilston, 1994). Therefore, at low protein concentrations, the lipid/protein complexes were prepared at lipid concentrations between 150 and 300 mM. A lipid concentration of 50 mM was used to obtain experimental conditions under which the lipid surface would be close to saturated. The lipid/protein stoichiometry at full saturation of the lipid surface was assumed to be 10 (Heinemann and Bilston, 1994; Görrissen and Marsh, 1986). calorimetric experiments were performed on a Calorimetry Sciences Corp. calorimeter (Provo, UT) at heating rates of 10°C/h and were corrected for the time response of the calorimeter. Calculations were performed on a commercial 66 MHz or 90 MHz IBM-compatible personal computer.
be determined from calorimetric experiments. The cooperativity parameter $\Delta \omega_{dl}$ is obtained by comparison of simulated results with the experimental transition halfwidth. Sugar et al. (1992, 1994) and Mouritsen and Biltonen (1992) showed that with this simple description the qualitative and the quantitative behavior of the heat capacity profile of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), small unilamellar vesicles (SUVs) can be represented.

### Interaction of lipid membranes with proteins

It is well known that the binding of peripheral proteins can affect the thermodynamic properties of a membrane. Usually the gel-liquid crystalline melting profiles are shifted and broadened. For integral proteins it is known from electron spin resonance experiments that lipids at the protein-lipid interface are restricted in their conformational mobility relative to their mobility in the bulk lipid phase (Marsh and Watts, 1982). Leaving aside the details of complex formation and the melting of lipid/protein complexes, one obtains the following scheme:

$$
\begin{align*}
G + P & \leftrightarrow L + P, \\
K_1 & \uparrow \downarrow K_2, \\
GP & \leftrightarrow LP, \\
K_3 & \uparrow \downarrow K_4,
\end{align*}
$$

with $G$ being the gel membrane, $L$ the fluid membrane, $P$ the free protein, and $GP$ and $LP$ the complexes of gel or fluid membranes with protein, respectively. The equilibrium constant $K_1$ determines the melting of the lipid in the absence of protein, and $K_4$ the melting of the lipid/protein complexes. The binding constants $K_2$ and $K_3$ describe the binding of protein to gel or fluid membranes. Because the free energy is a state function it follows that:

$$K_1 \cdot K_3 = K_2 \cdot K_4,$$

$$K_1 \neq K_4 \Rightarrow K_2 \neq K_3$$

This means that if the thermotropic melting transition shifts in temperature upon protein binding, then the binding constants to gel or fluid lipid phase must be different. This is equivalent to concluding that the binding reaction is responsible for the shift in $T_m$. The presence of bound protein can involve a change in the melting enthalpy, which is equivalent to a change in the binding enthalpy (Heimburg and Biltonen, 1994). These considerations are still valid if one uses a more detailed and complex scheme for protein binding and lipid melting as shown in Eq. 3 (scheme). The general results are not model-dependent.

Introducing the binding free energy changes $\Delta G_{P}^{\infty}$ and $\Delta G_{p}^{\infty}$, we describe the lipid-protein interaction through a differential binding free energy $\Delta G_{p} = \Delta G_{p}^{\infty} - \Delta G_{p}^{\infty} = \Delta H_p - T \times \Delta S_p$ defined per mol lipid. In the presence of protein the excess free energy is

$$
\Delta G_{ex} = n_l (\Delta H - T \Delta S) + n_{pl} \cdot \Delta \omega_{pl} + n_{pl} \cdot \Delta G_{pl},
$$

where $n_{pl}$ is the number of fluid lipids in contact with protein (Fig. 1).

The shift in the lipid transition at full saturation of the membrane surface with lipid is expressed by

$$
\Delta T_m = \frac{\Delta H + \Delta H_p}{\Delta S + \Delta S_p} - \Delta S
$$

using the pure lipid melting enthalpy $\Delta H$ and the melting entropy $\Delta S$.

If $\Delta S_p = 0$, than Eq. 4 can be simplified to $\Delta T_m = \Delta H_p / \Delta S$. Both the shift in the heat capacity maximum, $\Delta T_m$, and the change in the melting enthalpy, $\Delta H_p$, can be determined experimentally (Heimburg and Biltonen, 1994). An example is the complex of cytochrome c with DMPG (see Fig. 4 b), where $\Delta H = 6 \text{ kcal/mol}$ and $T_m = 297 \text{ K}$ (corresponding to $\Delta S = 20.2 \text{ cal \cdot mol}^{-1} \cdot \text{deg}^{-1}$). The binding of cytochrome $c$ results in a shift of the heat capacity maximum of $5^\circ C$ and a change in the melting enthalpy of $-4.3 \text{ kcal/mol}$ (therefore, $\Delta S_p = -14.6 \text{ cal \cdot mol}^{-1} \cdot \text{deg}^{-1}$). These experimental results mean that binding to the gel phase is thermodynamically favored. At the $T_m$ of the lipid protein/protein complex one obtains the differential binding free energy $\Delta G_{P}^{\infty} = 101 \text{ cal/mol lipid}$. The differential binding free energy, however, is temperature-dependent if $\Delta S_p \neq 0$. In the case of the binding of cytochrome c to DMPG, this leads to the intriguing consequence that at temperatures somewhat below the pure lipid melting temperature the sign of the differential binding free energy changes and the binding to the fluid phase becomes favored. This effect leads to a considerable broadening of the melting transition.

### Monte Carlo simulations

The melting behavior of lipid membranes has been described using Monte Carlo simulations following a standard Glauber procedure (Glauber, 1963). The probability for each lipid to switch states at a given temperature and in a given nearest neighbor environment was determined consecutively for all lipids in the matrix. The lipids were switched with this probability during sufficient Monte Carlo cycles to allow the lipid membrane to assume a representative random walk through phase space. The heat capacity was determined from the enthalpy fluctuations in the individual Monte Carlo steps using the fluctuation-dissipation theorem (Hill, 1960)

$$
C_p = \frac{1}{k \cdot T} \left( \frac{H - \langle H \rangle^2}{\langle H^2 \rangle - \langle H \rangle^2} \right).
$$

As we will show, the heat capacity calculated by Eq. 5 is, in the absence of protein, identical to the experimental heat capacity assuming an appropriate value for $\Delta \omega_{pl}$ (see Sugar
Equilibrium has been ascertained by a number of tests: the integral of the calculated $C_p$ function yields the calculated value of the average enthalpy; beginning with different initial configurations, the lattice yielded the same average enthalpy; and coarse graining over 10,000 Monte Carlo cycles indicated equilibrium had been obtained.

The Monte Carlo simulations were performed on a triangular lattice with $61 \times 61$ sites. The matrix $M$ is defined as

$$M(x, y), \quad -30 \leq x, y \leq 30$$

with periodic boundary conditions (e.g., $M(-31, y) = M(30, y)$). This lattice defines a torus with each lipid having six nearest neighbors. For each individual lipid the probability, $p_i$, to change from the gel state to the fluid state is

$$p_i = \frac{K}{1 + K},$$

$$K = \exp\left(-\frac{\Delta H - T \cdot \Delta S + \Delta n_{gl} \cdot \Delta \omega_{gl} + \Delta G_p}{R \cdot T}\right),$$

where $\Delta n_{gl} (-6 \leq \Delta n_{gl} \leq 6)$ is the change in the number of unlike, nearest neighbor interactions upon changing the lipid state, and $\Delta G_p$ is the differential binding free energy if a protein is situated on top (i.e., bound) of a lipid. $\Delta G_p$ is 0 if no protein is on top of the lipid. The probability of change from a fluid state to a gel state lipid is $p_g = 1 - p_i$. In the following simulations it has been assumed that $\Delta G_p$ is temperature-independent, which is equivalent to replacing $\Delta G_p$ with $\Delta H_p$. In the simulation a lattice point is selected randomly and the transition probability is calculated. A random number (RAN) between 0 and 1 is then generated and if the transition probability $\geq$ RAN the lipid is allowed to change state.

On top of the lipid matrix, a second matrix for all possible protein centers is defined. It is assumed that the protein concentration on the lipid surface is constant (canonical approximation). This approximation is valid whenever the number of membrane-bound molecules greatly exceeds the number in solution (Jorgensen et al., 1991), a condition that holds under most differential scanning calorimetry experiments. For example, differential scanning calorimetry experiments were performed at lipid concentrations of 50 mM, whereas the apparent dissociation constant has been estimated to be $10^{-3}$ to $10^{-6}$ M of lipid in both phases (Heimburg and Biltonen, 1994). For reasons of simplicity it has been assumed that possible midpoints of the proteins are identical with lipid positions (i.e., a $61 \times 61$ site-lattice), and that protein movement takes place via random movement of the protein center on that lattice. A number of $n_p$ protein molecules were randomly placed on that matrix. The proteins were chosen to have a radius $r_o$ and to cover $m$ lipids. In the simulations presented here usually $r_o = 2$ lipid diameters, resulting in $m = 19$ lipids per protein. In each Monte Carlo step associated with a change in state of a lipid, both the number of unlike nearest neighbor interactions $\Delta n_{gl}$ and whether a protein molecule is situated on top of the individual lipid molecule must be determined.

Initially, protein molecules are distributed randomly on the lipid surface. Their position, however, is changed by diffusion. The probability of the protein moving one position in a defined direction on the triangular lattice is controlled by the change of the number of fluid lipids underneath the protein upon movement. Therefore the probability of movement in the $i$th direction is given by

$$p_{in}(i) = \frac{K_{in}(i)}{1 + K_{in}(i)}, \quad K_{in}(i) = \exp\left(-\frac{\Delta n_{in}(i) \cdot \Delta \omega_{in}(i)}{R \cdot T}\right)$$

$$i = 1, 2, \ldots, 6$$

$\Delta n_{in}(i)$ is the change in the number of fluid lipids underneath the protein upon movement in direction $i$. Which direction to attempt to move was determined with a random number generator, and the decision to move was made with the use of Eq. 7. The decision to change an individual lipid state was made on the basis of Eq. 6 as described previously. Both Eqs. 6 and 7 fulfill the detailed balance condition (Mouritsen, 1989). In the simulations presented here it has been assumed that the diffusion of the proteins is on the same time scale as the transition time of individual lipids. Essentially the decision to flip a lipid is made as often as to move a protein molecule. This is, however, not of any importance, because the simulations are intended to be equilibrium calculations. It is clear that protein arrangement and lipid domain distribution are coupled to one another. In the case of the coexistence of gel and fluid domains the protein distribution will no longer be random if preferential protein binding exists.

RESULTS

Pure lipid membranes

A detailed analysis of the melting of DPPC-SUV on the basis of a two-state Ising model was given by Sugar et al. (1992, 1994) and Mouritsen and Biltonen (1992). The thermodynamic parameters for this system are the melting temperature, the melting enthalpy, and the cooperativity parameter, given by $T_m = 310.3$ K, $\Delta H = 8.7$ kcal/mol lipiod and $\Delta \omega_{gl} = 282.4$ cal/mol, respectively. In Fig. 2 the heat capacity profiles of the transition are given for two different values of the cooperativity parameters $\Delta \omega_{gl} = 282.4$ cal/mol and $310.3$ cal/mol, using the enthalpy and melting temperature of DPPC-SUV. The cooperativity of the transition is very sensitive to slight changes in the interfacial free energy $\Delta \omega_{gl}$. Increasing $\Delta \omega_{gl}$ results in the suppression of the coexistence of gel and fluid domains. Therefore the transition half-width decreases. In finite size scaling theory (Lee and Kosterlitz, 1991; Zhang et al., 1992; Corvera et al., 1993), a first order transition leads to two maxima in the distribution of membrane states at the heat capacity maximum. Fig. 3 shows the distribution of membrane states at the $T_m$ for four different values of the cooperativity param-
**FIGURE 1** Schematic picture of lipid-protein association. Local interactions $\Delta \omega_{gl}$ and $\Delta G_p$ for peripheral (left) and integral proteins (right) are indicated with solid and open arrows, respectively.

**FIGURE 2** Calculated heat capacity profile of a pure lipid membrane at two different values for the cooperativity parameter $\Delta \omega_{gl}$ ($\Delta \omega_{gl} = 282.4$ cal/mol and 310.3 cal/mol), $\Delta H = 8.7$ kcal/mol, and $T_m = 310.3$ K. Calculation were performed using a 61 * 61 site lipid matrix with periodic boundary conditions.

**FIGURE 3** Distribution of states during a Monte Carlo simulation at the heat capacity maximum, from top to bottom with $\Delta \omega_{gl} = 301.0$, 310.3, 319.6, and 328.9 cal/mol. All other parameters as in Fig. 2. The bottom curve has more than one maximum, indicative of a first-order transition. All other curves represent second order transitions (see text). Solid curves are fits with one-component or two-component (bottom) Gaussian distributions.

*Interaction of lipid membranes with peripheral proteins*

Consider now the binding of peripheral protein to the lipid system described in the previous section. The structural and thermodynamic nature of this interaction will not be considered in this simulation. The experiments shown or mentioned for comparison, however, were performed using charged globular proteins in complexes with negatively charged lipids. The lipid-protein interaction in these examples is predominantly of electrostatic origin. The simulations demonstrate the general effects of binding and allow one to make predictions of a general nature.

For the simulations it has arbitrarily been assumed that the peripheral protein has a diameter corresponding to five lipid diameters. Thus 19 lipids are covered by one bound protein molecule. This compares well with reported sizes of cytochrome c (10 lipids/protein; Heimburg and Biltonen, 1994; Görissen et al., 1986) and of phospholipase A$_2$ (~20 lipids/protein). Also, it has been assumed that the lipid concentration is sufficiently high to ensure that all protein is bound, independent of the state of the lipid. At closest packing, therefore, ~190 proteins can fit on the 61 * 61
lipid matrix. The differential interaction free energy \( \Delta E_p \) of protein binding was assumed to be 200 cal/mol (again being of the same order of magnitude for that of cytochrome c binding to DMPG (see above) with a preference of the protein for the lipid gel phase), a lipid cooperativity parameter \( \Delta \omega_{gel} = 310.3 \) cal/mol in approximate agreement with the DMPG transition halfwidth of \( \sim 1^\circ \) was used. The simulations of lipid-protein complexes were performed using different degrees of protein occupancy of the surface (30, 60, 90, and 120 proteins, corresponding to 16, 32, 48, and 64% of surface filling with protein).

Fig. 4a shows the calculated heat capacity profiles at the indicated degrees of surface occupancy. With increasing occupancy the heat capacity maxima are shifted to higher temperatures. The heat capacity curve shape becomes asymmetric and broadened. In the limiting case of complete surface coverage with protein, a sharp peak with similar cooperativity to that of pure lipid and a shift of \( \Delta T_m = (\Delta E_p / \Delta H) \times T_m = 7.1^\circ \) C is observed (not shown). Fig. 4b, by comparison, shows the calorimetric behavior of cytochrome c complexes with DMPG. Although in this system the assumed parameters are slightly different (\( \Delta H = 6 \) kcal/mol, \( T_m = 297 \) K, \( \Delta G_p = 100 \) cal/mol, and \( \Delta H_p = -4.3 \) kcal/mol), behavior qualitatively and quantitatively similar to the simulations is observed. At low degrees of protein occupancy of the membrane surface, a broadened \( C_p \) curve with a maximum at the lower end of the transition is observed. In contrast, at high degrees of surface occupancy the maximum is located at the high temperature end of the transition. This is due to the higher number of lipids with the tendency to melt at higher temperatures being in direct contact with protein.

As noted above, the incorporation of an interfacial free energy term \( \Delta \omega_{gel} \) leads to the formation of gel or fluid lipid domains in the proximity of the transition midpoint. This can be seen in Fig. 5, demonstrating the distribution of gel (black) and fluid lipids (white dots). It is shown at three different temperatures below, within, and above the heat capacity maximum of the transition of lipid complexes with 60 proteins per bilayer. The condition of the differential binding to gel and fluid lipids leads to an accumulation of the protein on the gel domains. Fig. 5 shows also the distribution of the peripheral proteins on the lipid lattice. The clustering of the protein occurs in the phase coexistence region and is a necessary consequence of the binding-induced shift of the heat capacity profile (Fig. 5, center). In the single phase regions (Fig. 5, top and bottom) the protein distribution is random.

Conversely, gel domains predominantly form in the proximity of bound protein molecules. To quantify this aggregation effect, a cluster parameter \( C(T) \) describing the mean distance of closest protein is defined as

\[
C(T) = \frac{d_{\text{random}} - d(T)}{d_{\text{random}} - d_{\text{min}}}, \tag{8}
\]

FIGURE 4 (a) Calculated heat capacity profiles of complexes of lipids with peripheral proteins, with increasing degree of membrane surface saturation. (Top to bottom) 0, 30, 60, 90, and 120 proteins, corresponding to 16, 32, 48, and 64% of surface filling. Parameters were: \( \Delta H = 8.7 \) kcal/mol, \( T_m = 310.3 \) K, \( \Delta \omega_{gel} = 310.3 \) cal/mol and \( \Delta E_p = 200 \) cal/mol, performed on a 61 \times 61 lipid matrix with periodic boundary conditions. (b) Calorimetric heat capacity curves of cytochrome c complexes with DMPG. Protein concentration corresponded to a surface filling of 15, 30, 50, and 100%. Experimental values for melting enthalpy, transition temperature, and differential binding free energy were \( \Delta H = 6 \) kcal/mol, \( T_m = 297 \) K, and \( \Delta E_p = 100 \) cal/mol (at \( T = 302 \) K).
Interaction of lipid membranes with integral proteins

The model for peripheral proteins presented in the previous paragraphs can be used to approximate a special case of the

where \( d_{\text{random}} \) is the average distance between two neighboring proteins on the lipid surface. \( d_{\text{min}} \) is the closest possible distance (defined by the protein radius) and \( d(T) \) is the mean closest distance in the presence of differential binding, determined experimentally during the simulation.

\[ C(T) = 0 \] for a random protein distribution and \[ C(T) = 1 \] in the case of complete protein aggregation.

The temperature dependence of the cluster parameter \( C(T) \) for the four lipid/protein ratios in Fig. 4a is given in Fig. 6. The aggregation is a maximum close to the temperature where gel and fluid lipids coexist in a 1:1 ratio. With higher saturation of the membrane with protein, the maximum of the aggregation curve shifts to higher temperatures in agreement with the shift in the heat capacity curves (Fig. 4a). For reasons of thermodynamic consistency the binding of cytochrome c to DMPG shown in Fig. 4b must also aggregate in the phase transition region. This has important implications for the distribution and, possibly, the function of peripheral proteins in general (see Discussion). The shift to higher temperatures follows from choosing a differential binding free energy \( \Delta E_\text{p} \) that is >0 and represents a preference of the protein for the gel phase. Assuming a value <0 (preference of the protein for the fluid phase) will lead to a shift in the heat capacity maximum to lower temperatures. Examples for both cases can be found in the literature (see Discussion).

**FIGURE 5** Peripheral proteins distributed on a lipid matrix at 32% surface saturation with proteins at temperatures below, within, and above the lipid melting transition and the fractions of fluid lipid, \( f \), indicated. (Black) Gel lipids; (white) fluid lipids. Protein molecules are shown as circles. The unit cell of the simulation is indicated by the rhombic box in the center of each part. The distribution is random below and above the heat capacity maximum and is highly nonrandom in the coexistence range of gel and fluid lipids. The simulation used periodic boundary conditions. Parameters as in Fig. 4a. Note that some lipids are covered by protein molecules and that in the center snapshot the proteins are predominantly situated on gel lipid domains.

**FIGURE 6** Protein cluster parameter defined through Eq. 8, demonstrating the effect of domain formation on the protein distribution at the lipid/protein ratios and thermodynamic parameters as in Fig. 4a. Symbols are the same as in Fig. 4a. The aggregation exhibits a pronounced maximum close to the heat capacity maximum.
interaction of lipids with integral proteins. In this special case we assume that the membrane-spanning part of an integral protein is equivalent to a core of either gel or fluid lipid. This is identical to assuming for a peripheral protein \( \Delta G_p = \Delta E_p = \pm \infty \). If, e.g., \( \Delta E_p = +\infty \), the protein core will be "gel-like" and interact with the lipids in the same manner as a gel lipid. The lattice points in this part of the matrix will always be in one fixed state (gel- or fluid-like) and not be considered as part of the lipid bilayer. Therefore they do not contribute to the statistical averaging of the thermodynamic observables. Lipid state changes will be modeled as before, and diffusion is modeled as with the peripheral protein (Eq. 7). This simplification leads to further reduction of the number of parameters, because no discrete value for \( \Delta E_p \) is assumed. All integral proteins will induce very similar heat capacity curves of lipid melting that depend only on the properties of the pure lipid (\( \Delta H, T_m \) and \( \Delta \omega_p \)) and the size of the protein. The interface between the integral protein core and the lipid in this description is similar to the interface between gel and fluid lipids.

Heat capacity curves for integral proteins with a diameter corresponding to five lipid diameters are shown in Fig. 7, using the parameters similar to those used for the peripheral protein and are indicated in the figure legend. Four different lipid/protein stoichiometries are shown. As with the peripheral proteins the heat capacity profiles are broadened and shifted to higher temperatures. The shift to higher temperatures in the simulations presented in Fig. 7 follows from \( \Delta E_p \) being \( >0 \). Taking a value \( <0 \) will lead to a shift in the heat capacity maximum to lower temperatures. Both can be found in the literature (see Discussion). For all protein concentrations, in contrast to membranes with peripheral proteins, the heat capacity maximum is situated at the lower end of the transition.

For a representative case, three snapshots of the simulated matrix are shown in Fig. 8 for temperatures below, on and above the heat capacity maximum. It can be seen that the integral proteins aggregate maximally in the fluid phase rather than in the lipid transition range. This is because integral proteins produce a permanent interface with the lipid chains, whereas an interface between lipid domains containing peripheral proteins exists only within the lipid phase transition. The aggregation behavior of integral proteins is given in Fig. 9 in terms of the aggregation parameter \( C(T) \), defined in Eq. 8. The aggregation starts in the lipid transition range and reaches a maximum close to a value of one above the transition.

The characteristic different shape of the lipid transition in the presence of peripheral and integral proteins is a consequence of the different aggregation behavior of the proteins. This difference is the result of assuming \( \Delta E_p = \pm \infty \) for the integral protein. Therefore, experimental heat capacity curves for lipid/protein interaction not only allow a distinction between peripheral and integral proteins; they also make it possible to predict aggregation of the proteins as a function of the membrane state.

**DISCUSSION**

In the previous sections a simple model for the interaction of peripheral and integral proteins with lipid membranes has been presented. It is based on a two-dimensional Ising model, allowing for only two states of the lipid, gel and fluid (Marsh et al., 1977; Mouritsen and Biltonen, 1992; Sagar et al., 1992, 1994). The model leads to the predictions of experimental differences between integral and peripheral proteins and to important predictions about the distribution of the proteins on or in the lipid matrix. We will argue below that this might have important relevance for the function of some proteins.

The introduction of an interfacial free energy \( \Delta \omega_p \) leads to the formation of lipid domains in the phase transition region. Although in single lipid bilayers there is so far no proof for this, this assertion is in agreement with the results of a number of other theoretical predictions (Mouritsen, 1991; Mouritsen and Biltonen, 1992; Zhang et al., 1992; Mouritsen et al., 1992; Mouritsen and Jorgensen, 1992) and experimental heat capacity data (Biltonen, 1990). In single lipid monolayers domain coexistence has been shown by fluorescence microscopy (Grainger et al., 1989, 1990).
lipid mixtures domain coexistence has been indirectly shown through FRAP (fluorescence recovery after photobleaching) experiments (Almeida et al., 1993; Vaz et al., 1989, 1990).

The interaction with integral proteins has been investigated theoretically by various groups. Based on a 10-state lipid model of Pink and Chapman (1979) and Pink et al. (1980), lipid-protein interactions were modeled assuming parameters related to attractive van der Waals forces between the lipids and the proteins and repulsive forces due to hydrophobic mismatch (Mouritsen and Biltonen, 1992; Sperotto et al., 1989, 1991; Sperotto and Mouritsen, 1991, 1993; Fattal and Ben-Shaul, 1993). This results in a net interfacial free energy difference between the lipids and the proteins and predicts the possibility of aggregation of integral proteins in the absence of protein-protein interactions. In particular, the simulations of Mouritsen and Sperotto (1992) showed that little aggregation occurred in the lipid phase thermodynamically preferred by the protein, but as the melting temperature was approached lateral density fluctuations induced the beginning of aggregation. Well into the phase coexistence region, massive aggregation occurred. Our results are consistent with those of Mouritsen and Sperotto (1992). In a related study, Zhang et al. (1993) calculated the phase diagram and heat capacity function for a pure lipid system undergoing a first-order phase transition at various compositions of an integral polypeptide that was assumed to occupy a site of the size of one lipid chain. The calculations predicted a closed phase-coexistence loop and a heat capacity function that develops a broad shoulder on the low temperature side and broadens and is shifted to lower temperatures as the concentration of peptide is increased. In addition, significant wings on the heat capacity function and
a specific heat peak outside the phase coexistence region (i.e., beyond the critical point) are observed. These results are similar to what we would obtain for an integral protein if \( \Delta E_n = -\infty \) (cf. Fig. 7).

It should be pointed out that the lipid composition at the lipid-protein interface is allowed to vary in the simplified model used here. The thermodynamic ability to do so is dictated only by the magnitude of \( \Delta \omega_{\text{lip}} \). Our results apply only to equilibrium configurations and do not address the question of whether lipids at the lipid-protein interface are motionally restricted as observed on the electron spin resonance time scale (Marsh and Watts, 1982) or rapidly exchanging as on the NMR time scale (Watts, 1993).

Although the model used by Mouritsen and co-workers contains specific structural detail, the two-lipid state model used in this paper has several advantages, including ease in the calculation of the heat capacity curve. The reduction of the model to two lipid states as used in this paper has two advantages: the model is very transparent, and it never contains more than four or five parameters, all of which are directly deducible from the experimental transition curves. The main reason of the limited cooperativity of the membrane transition is the finite interfacial energy between unlike nearest neighbor (defined by \( \Delta \omega_{\text{lip}} \)), which results in the formation of domains. The physical basis of this problem is the same in the 10-state Pink model (Pink and Chapman, 1979; Pink et al., 1980) and any Ising model with nearest neighbor interactions. No qualitatively different results therefore are to be expected.

Peripheral proteins induce a shift in the lipid transition curve to either higher or lower temperatures. Cytochrome \( c \) shifts the transition curve of DMPC to higher temperatures (Heimburg and Marsh, 1993; Heimburg and Biltonen, 1994; Fig. 4 b). Myelin basic protein induces a shift to lower temperatures in 1,2-dimyristoyl-sn-glycero-3-phosphaditylserine (DMPS) (Ramsay et al., 1986). In both cases the transition enthalpy is reduced significantly by >50% of the total heat. The shift in the transition curve is, on thermodynamic grounds, equivalent to a difference in the interaction free energy of the proteins with gel and fluid lipid. If a peripheral protein has more than one possible structural state, differential binding to the lipid may shift this equilibrium. If no shift in the heat capacity curve can be observed, no differential binding takes place and no lipid induced change in the protein function will be expected. Cytochrome \( c \) may serve as one example for biological relevance of this concept. It has been shown for cytochrome \( c \) that two functionally different states of the protein coexist on membranes (Hildebrandt et al., 1990; Heimburg et al., 1991; Heimburg and Marsh; 1993), both with different redox potential and different tertiary structure. According to the heat capacity curves in Fig. 4 b differential binding takes place in complex formation with DMPC membranes. It has been observed that lipid melting induces a shift in the equilibrium of these states and therefore affects the function of the protein. Thus thermodynamic consistency between protein-induced changes in lipid melting and temperature-induced changes in redox potential is observed.

Another point of potential relevance is the aggregation of peripheral proteins in the phase transition region. Phospholipase \( A_2 \) is a peripheral enzyme that hydrolyses lipids. It has been suggested that its activity is linked to dimerization or aggregation of the protein on the bilayer surface (Romero et al., 1987; Bell and Biltonen, 1989; Biltonen, 1990). Its activity is maximal near the phase transition of DPPC membranes (Biltonen, 1990; Lichtenberg et al., 1986; Op den Kamp et al., 1975), in agreement with the aggregation behavior predicted by the model presented here. Additionally, the activity maximum is shifted to lower temperatures in correlation with the number of proteins bound, which is consistent with the predicted shift in the aggregation maximum upon higher protein occupancy of the lipid surface (Fig. 6).

The melting enthalpy with cytochrome \( c \) on DMPC membranes is 1.7 kcal/mol compared with a transition enthalpy of 6 kcal/mol in the absence of protein (Heimburg and Biltonen, 1994). This leads to a very temperature-dependent differential binding free energy and a considerable entropic contribution of \(-14.6 \text{cal mol}^{-1} \text{deg}^{-1}\). The origin of this high entropic and enthalpic contribution and the different value for different proteins is not quite clear. It may, however, be linked to the hydration of the lipid/protein interface, which will, to a large degree, be an entropy effect.

The treatment of integral proteins in this work is very similar to peripheral proteins. It is reduced to the two special cases where the protein interface with the lipids is similar to either a gel or a fluid lipid core and calculated heat capacity curves are given (Fig. 7). To completely describe integral proteins in terms of a two-state model, an explicit free interaction energy with both, gel and fluid lipids has to be included, thus introducing two more parameters. In much more generality this problem has been treated, e.g., by Sperotto and Mouritsen (1991) or Mouritsen and Sperotto (1992), although no heat capacity profiles were given in these works. The main difference as compared with peripheral proteins is that integral proteins have been assumed to have a permanent interface with lipid chains. In fact, peripheral proteins would demonstrate the same behavior as outlined for integral proteins if they bound infinitely better to one type of lipid over the other. This leads to a qualitatively different aggregation behavior in membranes and as a consequence to different heat capacity profiles of the lipid melting. Again, protein-induced shifts to higher and to lower temperatures have been reported. A convincing example is the band 3 protein of erythrocytes, which induces a shift to higher temperatures in 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) membranes and shows qualitatively a very similar thermodynamic behavior as in Fig. 7 upon increase of the protein concentration (Morrow et al., 1986). Thus, it would be predicted that band 3 aggregates in the fluid lipid state, which, indeed, has been observed. A second example is cytochrome \( b_5 \) (Freire et al., 1983), which induces a shift to lower temperatures in DMPC and also shows qualitatively similar behavior to that
presented in Fig. 7. The different influence of the two proteins is probably an effect of different hydrophobic matching and does not lead to as large a change in the melting enthalpy of the lipids as in complexes with peripheral proteins. Furthermore, in a recent publication by Zhang et al. (1995), an example was given for the protein concentration-dependent effect of the heat capacity profiles of an integral peptide on distearyl phosphatidylcholine (C:18) membranes that is very similar to that calculated in Fig. 7. In this example a shift of the Cₚ profiles to lower temperatures was induced, indicating aggregation of the peptide in the gel phase. Another important example is bacteriorhodopsin, which exhibits aggregation in some lipids (Lewis and Engelman, 1983).

The functional relevance of clustering of integral proteins may be demonstrated by alamethicin, which forms pores consisting of aggregates of the peptide (Gennis, 1989). Recent experiments have shown that permeability of this pore can be linked to the probability of the lipid-forming non-bilayer phases (measured in terms of an anisotropic radius of curvature). This has been attributed to the formation of pores of various sizes (Vodyanoy et al., 1993; Keller et al., 1994). Woolley et al. (1994, p. 6850) argue that the channel activity of alamethicin pyromellitate “may be controlled by the process of self-association.” Pores of various sizes have earlier been observed by Hanke and Boheim (1980). In a paper by Boheim et al. (1980) the conductivity in a single lipid membrane was zero in the fluid state and non-zero in the gel phase. The dependence of the conductance as a function of temperature was similar to aggregation behavior predicted for integral proteins with a preference for the fluid lipid phase. It appears likely that alamethicin conductance is regulated by aggregation equilibria of the single peptide in membranes, which, as shown above, is regulated by membrane state. This might be of considerable importance for the conductance of many pores (see also gramicidin A; Huang, 1986: Cornell et al., 1989; Elliott et al., 1983).

An influence of lipids on protein behavior has also been demonstrated for a number of other proteins, such as the integral proteins αβ-hydroxybutyrate-dehydrogenase (Cortese and Fleischer, 1987), the transferrin receptor (Kurte et al., 1990), and the peripheral enzyme PKC (Nishizuka, 1984, 1986; Orr and Newton, 1992a,b). Recently, Yang and Glaeser (1994) have shown that the PKC substrate, the MARCKS peptide, induces clustering in membranes composed of phosphatidylcholine and phosphatidylserine. This domain formation results in substrate domain formation and is related to enzymatic activity.

As shown in this paper, the interaction of single lipid membranes and proteins can be described in terms of a simple physical model that leads to clustering of both proteins and lipids. This simple equilibrium model allows description of the thermodynamics of the interactions and straightforward extension of these kinds of studies to lipid mixtures that are more relevant for biological systems. Although we have used the gel-liquid crystalline transition in pure lipid system to generate unlike lipid-lipid interfaces, the results obtained are applicable to binary mixtures of lipids where composition rather than temperature is the appropriate variable. The resulting reciprocal thermodynamic relations may make it possible to understand the activation of proteins such as PKC on more complex membranes in terms of models that contain only experimentally accessible parameters with well defined thermodynamic meaning.

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