Interaction between proteins localized in membranes

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ABSTRACT We present a conceptual framework for evaluating the effect on the self-association of proteins in membranes due to (i) the presence of other proteins at high concentrations (excluded volume effect) and (ii) the high concentration and preoriented state of the reactive species. We have calculated the magnitude of such effects using plausible values for the concentrations of proteins in membranes, for the degree to which proteins may tilt and move vertically, and for their dimensions. Compared to the association of monomers tumbling freely in an experimentally realistic volume, we calculate that these factors alone can increase the likelihood of forming dimers 10⁵-fold and of forming trimers and higher oligomers many orders of magnitude greater. We discuss the implications of our calculations for experimental manipulation of membrane proteins, for biosynthetic assembly of multisubunit membrane proteins and formation of membrane lesions by assemblies of exogenous proteins, and for the activation of cellular events induced by the interaction of membrane receptors with themselves or with other membrane proteins.

Interactions between membrane-bound proteins play a significant role in a variety of cellular phenomena, including the transduction of signals across membranes, the transfer of membrane proteins between the plasma membrane and internal organelles, and the assembly of oligomeric protein structures. We wish to call attention to three generally unappreciated factors that can lead to a substantial enhancement of the extent of protein association in a membrane relative to that in solution.

(i) Membrane protein molecules occupy a substantial fraction of the total volume of biological membranes and as a result exclude a significant fraction of this volume to each other. Studies of protein association in vitro are conventionally carried out in solutions in which volume exclusion is neglected.

(ii) Integral membrane proteins have a preferred orientation relative to the plane of the membrane, whereas proteins in solution are randomly oriented.

(iii) Concentrations of membrane proteins are conventionally defined with respect to the entire volume of the membrane or cell suspension. However, the local concentrations of these proteins, which determine their thermodynamic activities, are substantially greater.

The purpose of this communication is to present simplified quantitative models for the effect of each of these factors upon association equilibria in membranes relative to solution and, by means of these models, to estimate the magnitude of effects that one might encounter under a variety of experimental conditions.

Model Membrane

The effects we wish to quantify depend upon the composition of the membrane and the size and shape of the membrane components. Thus, it is necessary at the outset to specify a standard physical representation of an "average" biomembrane, which, although necessarily oversimplified, provides a starting point for calculation. We assume an average integral membrane protein that is transmembrane, has a molecular weight of 50,000 (1), and is a rigid cylinder with a diameter of 4 nm and a height of 5 nm. We assume 20,000 such protein molecules per μm² of membrane, the remaining space being occupied (in the bilayer) by 2 million molecules of phospholipid and 1 million molecules of cholesterol (1). These values as well as the values for the surface areas of phospholipids and cholesterol—0.63 nm² and 0.35 nm², respectively (2–5)—can be used to calculate the fractional occupancy of the membrane surface area by protein. The value is 0.25. The representation is depicted schematically in Fig. 1.

Model Association Reaction

We shall calculate the effect of each of the factors mentioned in the Introduction upon the apparent equilibrium constant, Kₙ, for the formation of a single n-mer from n monomers:

$$nP \rightleftharpoons P_n \quad K_n = \frac{[P_n]}{[P]^n}$$

[1]

Effect of Volume Exclusion in the Membrane

Theoretical and experimental studies of the effect of excluded volume in macromolecular solutions indicate that when the fraction of solution volume occupied by macromolecules exceeds a few percent, equilibrium constants characterizing interactions between macromolecules may be markedly different than in the limit of high dilution (6, 7). The fraction of available volume occupied by cylindrical protein molecules in the previously defined model membrane is equal to the fraction of surface area occupied by the circular cross sections of these molecules. Thus, we employ a simplified theoretical model, described in Appendix A, which is a two-dimensional variant of the scaled particle model used previously to explore the effects of volume exclusion in solutions containing molecules freely moving in three dimensions.

To utilize this model we must specify the stoichiometry of the reaction to be examined and the relative size, shape, and fraction of surface area occupied by each membrane protein, including reactants, products, and unreactive species. Using Eq. A5 we may then calculate the value of correction factor for nonideality denoted by Fᵥₑₓᵣ, which is defined as follows: let Kᵥₑₓᵣ be the value of the equilibrium constant for the reaction measured under conditions such that all protein species taken together occupy a negligible fraction (1%) of the membrane surface area. Then, the value of the equilibrium constant measured under conditions such that all protein species taken

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together occupy a nonnegligible fraction of membrane surface area will be given by

$$K_n = \Gamma_{exc} K_n^*.$$  \[2\]

We first consider the effect of the presence of a single class of cylindrical "inert" (i.e., non-self-associating) proteins occupying a significant fraction of membrane surface area upon \(K_n\). The dependence of \(\Gamma_{exc}\) upon fractional area occupancy, calculated for the case that the circular cross-sectional area of monomer \(a_1\) is equal to that of the inert species \(a_{inert}\), is plotted in Fig. 2A for several values of \(n\). The same dependence, calculated for the case that \(a_1 = 10a_{inert}\) is plotted in Fig. 2B.

The following qualitative conclusions may be drawn from the results shown in Fig. 2: (i) increasing the fractional occupancy of membrane surface area by proteins enhances the tendency of dilute species to self-associate; (ii) the greater the number of molecules that associate to form an individual aggregate, the greater the degree of enhancement for a given fractional area occupancy; (iii) self-association of larger species is enhanced to a greater degree at a given area occupancy than self-association of smaller species.

Depending upon the extent of self-association (i.e., the number of monomers per oligomer) and upon the relative sizes of the inert and self-associating species, the equilibrium constant for self-association may be increased by one or more orders of magnitude at a fractional area occupancy of 0.25—i.e., at the fractional area occupancy characteristic of our model cellular plasma membrane.

**Effect of Restricted Mobility**

The entropy decrease associated with aggregate formation will be different for proteins confined to a membrane than for proteins in free solution. The magnitude of this difference can be approximated by calculating the molecular partition function for a particle in free solution and comparing it to that of the surface-bound particle. In these calculations, only the rotational and translational contributions to the free energy of the assembly process are considered—that is, solvent–solute interactions are neglected and the proteins are assumed to rotate and translate freely except for the restrictions on mobility imposed by the bilayer, which are modeled as infinitely high potential energy barriers (8). Vibrational and electronic contributions to the partition function are assumed to be independent of the rotational and translational mobility.

We also assume that the interacting species are asymmetric, having localized features such as that indicated by the shaded area in Fig. 1.

Membrane proteins are biosynthetically inserted into bilayers with a preferred orientation, and this anisotropy is maintained by the hydrophilicity of protein domains exposed to the aqueous solution relative to the lipid membrane interior. The orientation of the model protein relative to the plane of the membrane is specified by the angle between the cylindrical axis and an intersecting axis normal to the plane of the membrane. We shall model restrictions on the rotational mobility of the membrane protein by specifying that all orientations with \(\theta\) less than or equal to a maximum value, designated \(\Delta\theta\), are equally probable (Fig. 1). (If \(\Delta\theta = 180^\circ\), the rotation would be unrestricted.)

Using the appropriate molecular partition functions (Appendix B), one can calculate the free energy change for the association of \(n\) mol of monomer to form 1 mol of \(n\)-mer. The rotational contribution to the difference between the free energy of association occurring in the membrane and in solution is denoted by \(\Delta G_{rot}\) and is given by the Eq. B4.

One can define an enhancement factor, \(\Gamma_{rot}\) (similar to the factor \(\Gamma_{exc}\) defined in the previous section), as a ratio of equilibrium constants:

$$\Gamma_{rot} = K_{memb}/K_{soln} = [2/(1 - \cos \theta)]^{n-1}.$$  \[3\]

A plot of \(\Gamma_{rot}\) versus \(\Delta\theta\) is shown in Fig. 3 for dimerization (\(n = 2\)). Thus, the effect of restricted rotation on this reaction is to enhance formation of oligomer in a membrane relative to the same association occurring in free solution.

Membrane proteins are also restricted in translational mobility, which manifests itself as an increased local concentration. The fraction of the volume, \(V\), of a suspension of membranes or cells accessible to membrane-bound species will be denoted by \(f\). The local concentration will then be greater than the concentration averaged over the entire volume of the suspension (i.e., that measured experimentally) by a factor of \(1/f\). An apparent equilibrium constant
measured using these average concentrations of reactants and products is

\[ K_{\text{app}} = \frac{(P_n \times V)}{(P \times V)^n} \]

for \( P_n \) = \( n \)-mers and \( P \) monomers.

The equilibrium constant defined in terms of local concentrations is independent of \( f \) and will exceed the apparent equilibrium constant by a factor of

\[ \Gamma_{\text{trans}} = (1/f)^n - 1. \]

The value of \( f \) is calculated as follows: Let us consider a suspension of volume \( V \) containing \( n_c \) cells. Each cell has a membrane surface area of \( A_c \) and a volume of \( V_c \). The volume accessible to membrane proteins will be equal to the total membrane surface area times an allowed displacement normal to the plane of the membrane, \( \Delta z \) (shown schematically in Fig. 1). Then

\[ f = Q \Delta z, \]

where \( Q = n_c A_c / V \). We may estimate an upper limit for \( Q \) (denoted \( Q_{\text{max}} \)) in an experimental system by assuming that cells may be packed only until the intracellular volume is half of the total suspension volume, or \( V = 2n_c V_c \). This would be equivalent to a concentration of about 10^5 cells (radius, 10 \( \mu \)m) per ml. Using a value for the ratio \( A_c/V_c \) determined for baby hamster kidney cells (9), \( Q_{\text{max}} = 4.45 \times 10^{-6} \) \( \text{A}^2 \). The dependence of \( \Gamma_{\text{trans}} \) on \( \Delta z \) is plotted in Fig. 3 for a monomer–dimer association of membrane proteins on the surface of cells of the same dimensions under these conditions.

Several points may be made regarding the curves in Fig. 3. Plotting the enhancement factor on a logarithmic scale allows an easy assessment of the associated free energy at 37°. The free energy in kcal will be 1.4 times the logarithm of the enhancement factor. Both curves intersect the axis at large values of \( x \) (180° for \( \Gamma_{\text{rot}} \), 22,480 \( \text{A}^2 \) for \( \Gamma_{\text{trans}} \)), although they approach this lower limit at different rates. These values are outside the limits of what would make sense in our model membrane, indicating that both \( \Gamma_{\text{trans}} \) and \( \Gamma_{\text{rot}} \) are >0 and, therefore, that association of proteins is enhanced under any plausible conditions. At the other extreme, very small values of \( x \) give extraordinarily large enhancement factors (\( \Gamma \) goes to infinity as \( x \) goes to 0). It seems unreasonable to consider membrane proteins as totally immobile relative to the plane of the membrane. Klein et al. have analyzed the available data on the intramembrane segments of a variety of membrane proteins (10). Charged residues are found on either side of the hydrophobic sequence at an average distance of 1.7 residues from the membrane-spanning segment. Such hydrophilic residues will act as barriers to vertical movement of the peptide chain into the bilayer. This would then limit a displacement (\( \Delta z \)) of only about 5 \( \text{A} \) (1.7 residues \( \times \) (1.5 \( \text{A} \) angstrom/residue) \( \times \) 2). Using the same values and similar reasoning, the allowed tilt (\( \Delta \theta \)) is likely to be <10°.

From the above considerations we may conclude that the net effect of localization, orientational restriction, and volume exclusion is to enhance a self-association of proteins in membranes, relative to the same association of the same proteins at the same average concentration in solution. The extent of enhancement may be calculated using an effective equilibrium constant that is greater than the reference equilibrium constant by a factor \( \Gamma_{\text{trans}} \times \Gamma_{\text{rot}} \times \Gamma_{\text{exc}} \). Some sample calculations are given in Table 1. The greatest contribution of enhancement is apparently due to a concentration of the reactants.

The size of this latter contribution is of course determined by the choice of volumes and in that sense is extrinsic to the model. On the other hand, the magnitudes of \( \Gamma_{\text{exc}} \) and \( \Gamma_{\text{rot}} \) are intrinsic to the model since their values are determined by the system. By displaying the extrinsic and intrinsic contributions in a single table and calculating an overall enhancement factor, we do not wish either to obscure the contributions from the intrinsic factors or to artificially magnify these enhancements by applying the effect of dilution. Nevertheless, biochemists are wont to bring macromolecules into solution in order to purify or to analyze them or both. The compilation in Table 1 specifies the effect of such maneuvers on the interaction between membrane-associated components. Finally, we note that whereas some of the specific features of the model we have used affect the calculated enhancements, other features do not. We have listed the important and irrelevant variables in Table 2.

Discussion

We have developed a conceptual framework for evaluating the probability of protein–protein association when the reactive species are confined to a biomembrane rather than existing free in solution and have estimated the magnitude of three factors that affect such associations: the presence of other inert proteins, the high local concentration of the interactive species, and the limited rotational mobility of the

<table>
<thead>
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<th>Aggregate</th>
<th>( d_0/d_{\text{inert}} )</th>
<th>( \Gamma_{\text{rot}} )</th>
<th>( \Gamma_{\text{trans}} )</th>
<th>( \Gamma_{\text{exc}} )</th>
<th>Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimer</td>
<td>1</td>
<td>132</td>
<td>4300</td>
<td>2</td>
<td>1.1 ( \times ) 10^6</td>
</tr>
<tr>
<td>Trimer</td>
<td>1</td>
<td>1.7 ( \times ) 10^6</td>
<td>1.8 ( \times ) 10^7</td>
<td>4</td>
<td>1.2 ( \times ) 10^{12}</td>
</tr>
<tr>
<td>Dimer</td>
<td>10</td>
<td>132</td>
<td>4300</td>
<td>4</td>
<td>2.3 ( \times ) 10^6</td>
</tr>
<tr>
<td>Trimer</td>
<td>10</td>
<td>1.7 ( \times ) 10^6</td>
<td>1.8 ( \times ) 10^7</td>
<td>26</td>
<td>8.0 ( \times ) 10^{12}</td>
</tr>
</tbody>
</table>

The assumptions are (i) fractional area occupancy by all proteins = 0.25, (ii) \( \Delta \theta = 10° \), (iii) \( \Delta z = 5 \text{A} \), and (iv) reference volume equivalent to 10^6 cells per ml.
associating molecules that maintains them in their preoriented state.

Of course in any individual case, other factors, such as the attachment of the membrane proteins to cytoskeletal structures or the relative motion of individual domains in the monomer, can profoundly affect the rate and probability of association of membrane proteins with each other. Such factors cannot be quantified without a specific model. One of the uses of the calculations we have performed is to allow one to evaluate these other effects by excluding the contributions due to the more general factors we have considered.

The values listed in Table 1 show that the probability that interactive species will associate can be remarkably enhanced when the species are concentrated and preoriented in a membrane. In discussing our results we focus on three aspects: (i) the implications for experimental manipulations of membrane proteins, (ii) the implications for the biosynthetic assembly of multisubunit membrane proteins, and (iii) the implications for triggering of cell functions due to ligand-induced aggregation.

**Experimental Manipulations.** A common procedure for studying membrane proteins is to dissolve the membrane with mild detergents. It is apparent that this may disrupt protein–protein interactions of interest. In addition to the substantial dilution of the interacting species (11) the elimination of the enhancement due to the effects of excluded volume and of preorientation can markedly shift the equilibrium towards dissociation. This leaves entirely aside the effect of changing the solvent surrounding the proteins. If the sites by which the associating species interact are in the transmembrane segment, then of course the change in solvent can have additional disruptive effects. That at least in some transmembrane proteins, the sequence spanning the bilayer is tightly conserved (12, 13) suggests that these segments may be the locus of such interpeptide associations. Reincorporating the reactive species in liposomes can reestablish the hydrophobic milieu, the high local concentration of the reactive species, and their preoriented state. However, as commonly performed, such reconstructions do not take into account the excluded volume effect due to the presence of "bystander" proteins in the natural membrane. It can be seen from Fig. 2 A and B that the enhancement due to excluded volume in and of itself can become significant when higher order oligomers can form and if the interactive species themselves occupy a nonnegligible fraction of the space occupied by the total membrane proteins. For reconstituting such systems it may be useful to introduce a neutral "filler" protein into the reconstitution mixture to restore a more realistic milieu.

**Assembly of Membrane Proteins.** The effects of excluded volume, high concentration, and preorientation may provide significant driving forces for the biosynthetic assembly of oligomeric membrane proteins. Proteins may undergo a significant stabilizing conformational change when they become associated—sometimes dramatically so (14). It is likely that such a change requires an energy of activation that must be exceeded to achieve the new low energy state. The factors we have considered will shift the equilibrium of weakly interacting monomers to the associated state and help to drive this process. The polymerization of exogenous proteins inserted into membranes, such as occurs during lysis mediated by complement (15) and perhaps by cytotoxic lymphocytes (16), could be enhanced in an analogous manner.

**Transmembrane Signaling.** Certain cellular activities are initiated by the aggregation of receptors on the surfaces of cells. The aggregation of so-called "Fc receptors"—receptors that bind the Fc region of immunoglobulins—is a prime example (17). In this instance the cellular events are initiated when the immunoglobulins are crosslinked by a multivalent antigen, the receptors bound to the immunoglobulin thereby necessarily becoming aggregated also. Here, the multivalence of the ligand provides the driving force for association. In systems where a monofunctional ligand can apparently induce such associations (18, 19), the aggregation could result from ligand-induced conformational changes in the receptor that expose interactive sites or, alternatively, from ligand–ligand interactions. The effects we have considered can similarly influence ligands bound to the receptors—that is, ligand–ligand interactions might take place on the membrane that would not be observable in free solution. These interactions might indirectly drive the association between the binding proteins.

Finally, the effects we have analyzed are likely to be relevant to transmembrane transduction of signals. Here membrane receptors interacting with exogenous ligands initiate alterations in protein–protein interactions leading to the activation of membrane-bound enzymes that release products into the interior of the cell (20, 21). A rigorous quantitative analysis of such systems will require consideration of the factors we have analyzed here.

**Appendices**

**A. Calculation of the Correction of Equilibrium Constants for Nonideality.** The contribution of excluded volume to the energy of interaction between membrane proteins is estimated by treating the membrane as a two-dimensional solution of protein solute(s) in lipid solvent. (Technically one should therefore speak of excluded area rather than excluded volume.) A general reaction between an arbitrary number of solute species is depicted by

\[
\Sigma x_i X_i \rightleftharpoons \Sigma x_i X_i
\]

where \(x_i\) is the stoichiometric coefficient of species \(X_i\). The condition of chemical equilibrium is

\[
\Sigma \mu_i = \Sigma \mu_i
\]

\(\mu_i\) is the chemical potential of species \(X_i\), is given by

\[
\mu_i = \mu_i + RT \ln y_i c_i
\]

where \(\mu_i^0\) is a standard state chemical potential, which is independent of the composition of the system at constant temperature, \(R\) is the molar gas constant, \(T\) is the absolute temperature, \(c_i\) is the concentration of species \(i\) in units proportional to number density (for example, \(g/cm^2\)), and \(\gamma\) is a composition-dependent activity coefficient, which reflects the energetics of intermolecular interaction between molecules of \(X_i\) and all other solute species (6).
We define the apparent equilibrium constant for reaction A1 as
\[ K = \frac{\Pi c_i^f}{\Pi c_i^i}, \] \[ \text{products reactants} \] \[ \text{Eqs. 1-4} \]

Text Eq. 1 follows from Eqs. A2–A4, where \( \gamma_{exc} \), the nonideal correction factor, is given by
\[ \gamma_{exc} = \frac{\Pi y_i^f}{\Pi y_i^i}. \] \[ \text{reactants products} \] \[ \text{Eqs. 5-7} \]

The problem of evaluating the effect of volume (or area) exclusion upon the equilibrium constants of reactions involving solute molecules is thus reduced to the problem of evaluating each of the \( y_i \) as a function of composition. It is important to note that the \( y_i \) may depend upon the abundances of all solute components, not just those participating in the particular reaction studied. In the context of our model we find that the activity coefficients depend significantly only upon the concentrations of species occupying more than about 1% of membrane surface area.

The activity coefficient of species \( X_i \) may be generally represented by
\[ \ln y_i = \sum_j B_{ij} C_j + \sum_k B_{ik} C_k + \ldots, \] \[ \text{[A6]} \]

where \( B_{ij}, B_{ik}, \ldots \) are constants at constant temperature, whose values are determined by the potentials of interaction between any given pair, triplet, \ldots of solute molecules in the lipid solvent (22). However, the evaluation of \( \ln y_i \) by means of Eq. A6 is prohibitively difficult in any but extremely dilute solutions. For this reason, recourse is made to approximate theories of hard particle fluids, which have proven to be useful in the evaluation of the activity coefficients of proteins in concentrated (three-dimensional) solutions (6).

The present calculations were performed by using the scaled particle theory of Lebowitz et al. (9) for two-dimensional mixtures of hard disks, which is appropriate for our model of membrane protein molecules as cylinders whose cylindrical axes are parallel and whose centers of mass are coplanar. Consider a membrane containing a number density \( \rho_0 \) of proteins of cylindrical radius \( R_1 \), a density \( \rho_2 \) of proteins of cylindrical radius \( R_2 \), and so forth. According to the scaled particle theory, the activity coefficient of species \( i \) in this mixture of species is given by:
\[ \ln y_i = -\ln(1 - S_2) + [2S_1/(1 - S_2)]r_i + [S_0/(1 - S_2) + S_1/(1 - S_2)^2]r_i, \] \[ \text{[A7]} \]

where
\[ r_i = \Pi \sum_j r_j. \]

Since all species must be represented by cylinders (disks), the approximation has been made that an oligomer formed from \( n \) cylindrical monomers may be represented by a cylinder of cross-sectional area equal to \( n \) times that of a monomer.

B. Calculation of the Effect of Rotational Restriction. The Gibbs free energy, \( G \), for a collection of particles is a function of the canonical partition function, \( Q \):
\[ G = -kT \ln Q + PV, \] \[ \text{[B1]} \]

where \( P \) is the pressure, \( V \) is the volume, \( k \) is the Boltzmann constant, and \( T \) is the absolute temperature. Separating \( Q \) into its component molecular partition functions, one can derive an expression for the rotational contribution of each particle, \( q_i \), to \( Q \) (23, 24):
\[ q_i = (I_i L) c_i^{1/2} (2\pi kT/h^3)^{3/2} 4\pi^2 (1 - \cos \theta), \] \[ \text{[B2]} \]

where \( I_A, I_B, \) and \( I_C \) are the moments of inertia about the three principal axes, \( h \) is Planck's constant, and \( \theta \) is the angle described in the text and corresponds to one of the three Euler angles.

The rotational contribution to \( Q \) will be \( (q_i)^N \) for \( N \) particles. The difference in free energy between an association of \( n \) monomers to form one \( n \)-mer in free solution where \( \theta = \pi \) (180°) and in the membrane where \( \theta = \Delta \theta \), is given by:
\[ \Delta G = (G_{n-mer,\Delta \theta} - nG_{monomer,\Delta \theta}) - (G_{n-mer,180} - G_{monomer,180}). \] \[ \text{[B3]} \]

Therefore, for \( N = 1 \) mol, the rotational contribution will be
\[ \Delta G_{rot} = (n - 1)kT \ln(2/(1 - \cos \Delta \theta)). \] \[ \text{[B4]} \]