Attraction within the membrane

Forces behind transmembrane protein folding and supramolecular complex assembly

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Received July 16, 2002; revised September 18, 2002; accepted September 24, 2002

Biological membranes are fascinating two-dimensional micro-environments that exhibit unique solvent behaviours due to their varying lipid composition. Although many important bioenergetic and signalling events involve the transient or permanent assembly of membrane protein complexes, the characterization of the thermodynamic and kinetic properties behind this assembly is just beginning. In particular, the molecular forces that govern protein association within these structures remain poorly understood. An understanding of the docking of transmembrane proteins to supramolecular complexes, which will make possible the development of predictive computational tools, will require detailed knowledge of interaction forces at the atomistic or residue level. Here, I review current data on supramolecular complexes in membrane environments and make a tentative comparison between assembly processes in membranes and those driven by the hydrophobic effect in water. This comparison suggests that, in addition to being controlled by specific characteristics of the lipid molecules themselves, molecular assembly in the membrane milieu also depends more generally on the entropy of the lipid fraction.

Introduction

Biomolecular supracomplexes, which consist of two or more separate proteins that are transiently or permanently bound, are important regulatory elements in biological cells, and their formation is currently a topic of great interest. In fact, a spectacular scan of the yeast genome was recently carried out to identify new complexes (Gavin *et al.*, 2002). Unfortunately, for technical reasons, this study was limited to soluble complexes. Nevertheless, a number of membrane protein-containing complexes, including the nuclear pore complex and the endoplasmic reticulum Sec61p translocon, have been characterized by other means. In these two cases, the resulting complex is not just the

sum of its parts since assembly is necessary for the complex to function. This is in contrast to a number of bioenergetic systems like the 'photosynthetic unit' in *Rhodobacter sphaeroides* (composed of light harvesting complexes I and II, bacterial reaction centres and the cytochrome *bc₁* complex), which converts light energy into a cross-membrane pH gradient, two-dimensional bacteriorhodopsin (BR) lattices and supramolecular assemblies of complexes I, III and IV of the mitochondrial respiratory chain (Schagger and Pfeiffer, 2000). For these systems, assembly into complexes seems to be mechanistically favourable but not compulsory. Although it remains to be shown whether formation of supramolecular complexes is limited to certain membrane types, these studies suggest that organizing functionally coupled membrane proteins into complexes may be a general principle.

However, while it may be functionally advantageous to permanently assemble components of bioenergetic machineries into large complexes such as those listed above, the formation of such assemblies may, on the other hand, obstruct time-critical diffusion processes in membranes that are actually specialized for other purposes. Phospholipid membranes in plants and bacteria (20–90% protein) are generally highly dynamic systems, with the rate of lipid diffusion 100–1000 times higher than that of membrane proteins. Even though diffusion within cellular membranes is not completely free, due to two-dimensional compartmentalization of the membrane by proteins of the cytoskeleton and to reduced mobility in regions of higher viscosity, the mobility of membrane proteins can have a great impact on signalling efficiency. For example, in mice, phototransduction begins when photoexcited transmembrane rhodopsin (R*) and the GPI-linked G protein transducin diffuse within and on the disc membranes of retinal rod cells, collide and then bind to one another. In this process, the speed of diffusion of R* and transducin is restricted by an enormous number of quiescent rhodopsin molecules, as illustrated by the fact that reducing the content of the unexcited, speed-limiting rhodopsin

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by 50% in transgenic mice leads to an increase in signal intensity *in vivo* (Calvert *et al.*, 2001). This study established that the rate of phototransduction in the rod cell is determined by protein diffusion in the disc membrane rather than by the amount of protein capable of transducing the signal.

Energetics

Folding and assembly in a membrane environment are energetically very different from the same processes carried out in water because there is no hydrophobic driving force: the 'outside' and 'inside' surfaces of the transmembrane (TM) parts of membrane proteins are very similar. The bundling of TM helices, as part of the folding process of helical TM proteins, is likely to be governed by forces similar to those that drive the complex formation of TM domains belonging to separate membrane proteins (Figure 1A). Traditionally, the prediction of membrane protein structure has been considered a 'problem of physical chemistry' (White *et al.*, 2001), where physical influences include interactions of the polypeptide chains with water, one another, the bilayer hydrocarbon core, the bilayer interfaces and cofactors (White *et al.*, 2001). Structural and energetic perturbations of lipid-lipid interactions have rarely been considered.

According to the established two-stage model for membrane protein folding (Popot and Engelman, 1990), TM helices fold spontaneously after being incorporated into the membrane, and bundle into the final three-dimensional structure in the second step. This model was recently expanded into a four-step model (White *et al.*, 2001) involving interfacial partitioning, interfacial folding, insertion into the lipid bilayer and bundling within the lipid bilayer (see Figure 1B). These steps may take place at the water/lipid interface, in water or a combination of the two. Additional steps may involve the membrane-resident translocon, through which nascent peptide chains are delivered from the ribosome to the ER.

Regardless of the details of membrane insertion, every model that assumes preformation of α -helices requires them to bundle with other α -helices at some stage. The structural determinants of helix-helix packing were recently investigated in 14 transmembrane proteins with known structure (Adamian and Liang, 2001), and the authors found that these were different in membrane and soluble proteins. Packing between backbone atoms, for example between those of proximal Gly residues, seems to be more common in membrane proteins than in soluble proteins. Gly has the smallest side chain of any amino acid, thus allowing the closest contact between pairs of helices. Moreover, the peptide backbone of Gly can be distorted most easily from the canonical α -helical conformation, thus allowing non-standard orientations. Another study compared sequences and structural features of helices in TM versus soluble proteins (Bywater *et al.*, 2001). Residue types not typically associated with helix stabilization in soluble proteins, such as Gly, Pro, Ser, Thr, Asn, Ile and Val, were found to be relatively common in TM helices, which could potentially indicate differences in helix packing. However, the authors did not observe any geometric consequences of these sequence patterns: helix-helix crossover angles were similar in both types of protein, and there was no appreciable difference with respect to helical three-dimensional nearest-neighbour contacts, side-chain rotamer angles and peptide-bond angles when TM and globular proteins were

compared. The authors noted that TM helices are nevertheless subject to deformations that may be local or extend over almost the full length of the helix, and proposed that this phenomenon plays a role in helix bundle packing. NMR studies on the M2 channel-lining segments of nicotinic acetylcholine and N-methyl-D-aspartate (NMDA) receptors showed that hydrogen bonds and salt bridges, in which the hydrophilic face of the helices is orientated toward the interior of the bundle, are important for interactions between amphipathic α -helices (Opella *et al.*, 1999). Moreover, attractive forces may result from interactions between the hydrophilic external parts of the proteins, and the phospholipid composition of the membrane may also have an effect on membrane protein organization (Bogdanov *et al.*, 2002).

Predicting the three-dimensional structures of TM domains should, in principle, be simpler than predicting the structures of soluble proteins, because the bundling of the TM helices requires only a translational search in *x* and *y* dimensions of the membrane plane plus a rotational search along each helical axis. Given a suitable method for assigning energies to particular molecular geometries, a computer program could easily generate a large number of possible helix arrangements and calculate the structure with the most favourable energy. For soluble proteins, simple, yet powerful, energy parameterizations, so-called 'statistical potentials' between pairs of amino acids, have been generated by inverting the probabilities of occurrence at certain distances in the large number of known crystal structures in the Protein Data Bank. These have been used successfully, for example, in protein folding simulations and for ranking docked conformations of protein complexes. Unfortunately, the number of known membrane protein structures is too small to construct statistical potentials, and the number of known structures is not likely to increase very quickly over the coming years. Therefore, the only methods currently available for computing such interaction energies for TM proteins are approximate potentials based on qualitative insights (Fleishman and Ben-Tal, 2002) or molecular mechanics force fields, which rely on an atomistic description and are therefore computationally expensive to use. It is, for example, computationally impossible to perform an unrestricted conformational search in atomistic detail to construct structural models of transporter proteins with 12 TM helices. However, atomistic force fields have been used to perform numerous molecular dynamics simulation studies for smaller protein systems that contain only a few transmembrane α -helices (Biggin and Sansom, 1999), and structural models of TM proteins have been constructed using molecular dynamics simulations in which the relative orientations of the helices are forced to fulfill certain geometrical distance restraints (Sansom, 1995).

What might the lipid contribution to protein folding/association in the membrane be? Do the lipids form a simple matrix that passively accommodates protein rearrangements? This seems not to be the case, since binding sites specific for lipid molecules are found on the surface of integral membrane proteins. For example, a recent high-resolution crystal structure of the cytochrome *bc₁* complex from yeast revealed phosphatidylinositol- and cardiolipin-binding sites (Lange *et al.*, 2001). Also, specific lipids bound between subunits of supramolecular complexes appear to form a flexible interface between protein subunits, providing both binding energy to stabilize ionic interactions and also a flexible hydrophobic interface, thus reducing constraints

Fig. 1. Helix formation and bundling/association within membranes. (A) Schematic illustration of the forces that may lead to bundling of transmembrane helices in proteins with multiple helices (left), and to association of transmembrane helices belonging to different proteins (right). 1 represents protein:water, 2 protein:protein, 3 protein:bilayer core and 4 protein:bilayer interface interactions. (B) Depiction of the four-step model of membrane protein folding according to White *et al.* (2001). 1 represents interfacial partitioning, 2 interfacial folding, 3 membrane insertion and 4 helix bundling. Proteins are shown in blue, lipid molecules in orange and water molecules in red and grey.

on the types of amino acid residues that can be present at the interface and increasing the entropy of interaction. However, in addition to these kinds of protein-specific role, a consideration of the behaviour of proteins in water suggests that lipids are also likely to have a more general function, as outlined in the following section.

Analogy with the hydrophobic effect

The well-known hydrophobic effect is the driving force for the folding of soluble proteins in water, and for the association of hydrophobic patches on protein surfaces. According to the currently established view (Huang and Chandler, 2002; Southall *et al.*, 2002) the underlying mechanism changes from an enthalpy-driven effect at low temperature (i.e. preference for a small number of states with favourable energies) to an entropy (i.e. measure of disorder)-driven effect at higher temperatures (favouring assembly or disassembly depending on which provides a larger number of molecular conformations at roughly the same energy). In all environments, however, enthalpic elements—such as the attraction between solute and water molecules—are combined with complex entropic effects generated by water molecules in the close vicinity of the solute (see Figure 2A). Water molecules in contact with a hydrophobic surface are available to significantly fewer neighbouring water

molecules for hydrogen-bond formation than are their 'free' counterparts. This leads to a smaller number of energetically favourable orientations and results in an unfavourable entropy relative to that of the bulk water molecules that are free to rotate. Reduction of solvent-exposed hydrophobic surface, with a concomitant increase in solvent entropy, is therefore the driving force for protein folding and association in water.

Keeping this picture in mind, we return to helices and proteins dissolved in phospholipid bilayers (see Figure 2B). In 1972, the fluid mosaic model of biomembranes, in which all membrane components are proposed to float freely within the membrane, was established (Singer and Nicholson, 1972). Such a situation would be entropically very favourable, and NMR measurements and molecular dynamics simulations have now shown phospholipid molecules within pure lipid bilayers to be quite disordered. On the other hand, orientated lipids tightly bound to rigid TM proteins must be regarded as entropically confined (White and Wimley, 1999). So if entropic contributions are important, why should the various proteins of, for example, a photosynthetic unit, assemble into a well-defined quasi-rigid arrangement? Seen simply from the protein perspective, this process would be entropically very unfavourable. Protein complex formation, however, is not dependent on the entropy of the protein components alone, but rather on the entropy of the total system. Therefore, it is important to consider that reducing the lipid-exposed protein

Fig. 2. The hydrophobic effect and a potentially similar mechanism for protein assembly within membranes. (A) Binding of two proteins (green and grey) in water. Shown in dark blue on the left is the shell of water molecules in contact with protein (the first solvation shell). Binding is accompanied by a significant reduction in the contact surface between protein and water molecules—the ‘solvent-exposed surface area’. On the right side, the fine structure of water that surrounds the protein complex is illustrated. (B) Assembly of a membrane protein complex. The lipids in the first and second layers around the proteins are assumed to be entropically confined and are marked by black dots (76 when grey and green proteins are separated, and only 56 and 62 in the ‘tight’ or ‘lipid-mediated’ complexes).

surface, either completely or partially, means that a number of lipid molecules return to the lipid pool, thereby increasing their entropy, as shown in Figure 2B. (It is possible that a few lipid molecules remain bound at the interface between TM proteins, resulting in a ‘lipid-mediated complex’ reminiscent of soluble protein complexes, in which the interfaces often retain a few water molecules.) Lipid entropy may therefore be an important driving force for membrane protein folding and complex formation. A truly complex picture emerges when one considers the large number of different lipid species and composition present in biological membranes. The precise lipid character of each membrane may favour different extents of association, and studies in which lipid composition is varied may enable one to draw important conclusions about the energetics of these processes.

Experimental evidence

Measuring subtle entropic differences in protein association in membrane milieus is certainly an experimental challenge. To investigate the thermodynamic stability of the two-dimensional BR lattice, a naturally occurring two-dimensional crystal, residues at the BR–BR interface were replaced by smaller amino acids, either Ala or Gly (Isenbarger and Krebs, 2001; see Figure 3). Although most of the mutant lattices were destabilized as predicted, an I45A mutant was actually stabilized significantly, and the authors hypothesized that this might be due to increased lipid entropy. In another approach, the dimerization thermodynamics of the glycophorin A (GpA) TM helix was measured in detergent micelles (Fisher *et al.*, 1999) by detecting Förster resonance energy transfer between two fluorescent labels attached to the N-terminus of the TM helices. The free energy of GpA helix dimerization in SDS was found to result from favourable enthalpic and entropic terms, each contributing -4 kcal/mol. In light of the biophysical considerations outlined in Figure 2 and

the fact that ~ 400 Å² of lipid-exposed surface are buried upon dimerization, one would expect that most of the favourable entropy of binding can be attributed to the release of SDS molecules from the GpA interface to the entropically favourable pool of bulk SDS molecules.

Theoretical approaches

Disentangling the driving forces in complex systems should, in principle, be one of the realms of computer simulation methods. Work in the theoretical biophysics community, however, has concentrated so far on the diffusion behaviour of membrane proteins and on pattern formation of complex protein mixtures in membranes. It has not addressed the fine energetic and structural details of macromolecular complex formation. For example, a molecular dynamics study modelled the non-uniform distribution of the two photosystems, PSI and PSII, in the grana (PSI) and stromal lamellae (PSII) of chloroplast membranes by simulating a system in which individual proteins are modelled as spheres that interact with each other by pair-wise forces (Rojdestvenski *et al.*, 2002). The effective interaction between PSI or PSII particles was described as the sum of a repulsive electrostatic (Coulombic) force between the soluble domains of the photosystems, which carry negative charges of different magnitudes (Figure 1A), and of an exponentially decaying attraction that mimics the lipid-mediated interactions between their TM domains. Depending on the ion concentration of the solution, the strength of the repulsive forces between photosystem particles is assumed to be shielded (screened) to different extents and the ordering of photosystems within the membrane depends on a complex interplay between electrostatic and lipid-mediated interactions. Simulations in which the strengths of these interactions were varied revealed the complicated phase behaviour of the system—a quasi-crystalline phase of

Fig. 3. Amino acid substitutions that stabilize the two-dimensional bacteriorhodopsin (BR) lattice, possibly by increasing increased lipid entropy, as has been suggested by Isenbarger and Krebs (2001). Two BR proteins of a BR trimer are shown, with the A–G helices of each indicated. The substitution of Ala for Ile at position 45 of the interface between the interacting B and D helices of neighbouring monomers is known to stabilize these complexes.

randomly distributed PSI and PSII complexes at low ionic screening, a well-defined clustered state of segregated complexes at high screening (a ‘PSI-world’ and a ‘PSII-world’), and an intermediate ‘agglomerate’ phase in which the photo-systems tend to aggregate together without segregation into PSI or PSII clusters. In this study, the molecular nature of the lipid molecules was not considered, and possible entropic effects of these are therefore not accounted for.

In another example of a phase behaviour simulation, two-dimensional arrays of membrane proteins were studied by dynamic Monte Carlo simulations, a method in which proteins and two different sorts of lipid are explicitly modelled as hard rigid discs of equal size (Sabra *et al.*, 1998). These simulations predicted that two-dimensional array formation is promoted by having two different lipid species present in the membrane, one of which should interact more strongly with the protein than the other. Another study—closest in spirit to the entropy hypothesis presented here—examined the physical forces that determine the state of minimal free energy of the chlorophyll *b*-containing chloroplasts as manifested by grana formation (Chow, 1999). The author proposed that the ordering of thylakoid membranes and of intramembrane protein complexes is driven by an increase in the overall entropy of the system associated with an increase in diffusion volume for membrane and stromal components.

The structural nature of lipid-mediated interactions between small membrane proteins was recently examined using an integral equation formalism (Lagüe *et al.*, 2000) that predicts perturbations of the uniform lipid density in a pure lipid bilayer caused by one or more inserted membrane proteins modelled as rigid cylinders. A free energy profile is constructed by correlating fluctuations in the density of lipid molecule carbon pairs in molecular dynamics simulations of an unperturbed lipid bilayer. Currently, the method is able to predict general trends of non-specific lipid-mediated protein assembly—the calculations

showed, for example, that the average hydrocarbon density is perturbed over a distance of 2–2.5 nm from the edge of a TM cylinder—although it is not able to predict specific effects that relate to a particular protein surface or to a certain lipid mixture. In this model, entropic effects in the lipid fraction can be captured from the predicted variations of the lipid density. In future, approaches of this kind should allow the successful description of association processes in membranes, provided detailed modeling of lipid molecules is not required.

Perspectives

Although the attractive forces at work between TM helices and between different integral TM proteins are likely to be of a different nature from those between soluble proteins, increasing evidence indicates that the formation of supramolecular complexes in membranes may be accomplished in much the same way as the formation of supramolecular complexes of soluble proteins. It is suggested here that the lipid component of the membrane exerts entropic control over membrane protein assembly, playing a role similar to that of the water molecules in producing the hydrophobic effect. However, our understanding of assembly in membrane environments, so far, is merely at the level of individual observations. Clearly, what are needed are quantitative experiments where system parameters are changed one at a time. Only then will we be able to better understand and disentangle the complex and interdependent energetic contributions. One recent example of such quantitative experiments is an Ala-scanning mutagenetic study that examined the sequence dependence of GpA dimerization (Fleming and Engelman, 2001). A conserved hierarchy of stability was observed for the different GpA mutants when inserted into three chemically different hydrophobic membrane environments. This suggested that the protein–protein component of the overall

energy may be experimentally separable from the protein–lipid and lipid–lipid energy components. Developments of this kind should stimulate on-going efforts to develop theoretical docking and simulation tools, so that they may enhance our understanding of membranomics.

Acknowledgements

I thank Markus Elsner and Rene Staritzbichler of my group for their input and the anonymous reviewers for their suggestions. This work has been supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 472-Teilprojekt 23).

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Volkhard Helms is the recipient of an EMBO Young Investigator Award

DOI: 10.1093/embo-reports/kvf245