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Method for measuring the unbinding energy of strongly-bound membrane-associated proteins

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ABSTRACT

We describe a new method to measure the activation energy for unbinding (enthalpy ΔH^*_u and free energy ΔG^*_u) of a strongly-bound membrane-associated protein from a lipid membrane. It is based on measuring the rate of release of a liposome-bound protein during centrifugation on a sucrose gradient as a function of time and temperature. The method is used to determine ΔH^*_u and ΔG^*_u for the soluble dengue virus envelope protein (sE) strongly bound to 80:20 POPC:POPG liposomes at pH 5.5. ΔH^*_u is determined from the Arrhenius equation whereas ΔG^*_u is determined by fitting the data to a model based on mean first passage time for escape from a potential well. The binding free energy ΔG_b of sE was also measured at the same pH for the initial, predominantly reversible, phase of binding to a 70:30 PC:PG lipid bilayer. The unbinding free energy (20 ± 3 kcal/mol, 20% PG) was found to be roughly three times the binding energy per monomer, (7.8 ± 0.3 kcal/mol for 30% PG, or est. 7.0 kcal/mol for 20% PG). This is consistent with data showing that free sE is a monomer at pH 5.5, but assembles into trimers after associating with membranes. This new method to determine unbinding energies should be useful to understand better the complex interactions of integral monotopic proteins and strongly-bound peripheral membrane proteins with lipid membranes.

1. Introduction

Lipid bilayers separate cells from the environment and also partition cells into different compartments. While some proteins span the entire lipid bilayer, many important functions are carried out by proteins that bind to only a single leaflet of a lipid membrane [1–6]. These proteins play important roles in numerous cellular functions including signaling, synthesis and breakdown of molecules, trafficking, fusion and budding of viruses, and neurotransmitter release, among others. Those functions are likely to be affected by binding affinities, residence times at the membrane, conformations, and unbinding energies. As an example, localization of ubiquitous amphitropic proteins [2] to either the aqueous phase or to a cell membrane is regulated through various mechanisms that alter the strength of the protein–membrane interaction. Similarly, anchoring energies are crucial in protein-induced membrane bending that occurs in vesicle formation [7,8] or membrane fusion [6]. As a further example, subcellular localization of FYVE zinc finger domains may depend on a threshold level of binding [9]. To date such systems have been studied mainly through measurements of equilibrium binding constants. However, that data provides only a partial understanding, as proteins associate irreversibly in many cases.

Proteins that associate with only a single leaflet of a lipid membrane are classified as integral monotopic proteins and peripheral membrane proteins [5]. Integral monotopic proteins are permanently attached to one side of a lipid membrane. Modes of interaction that contribute to strong attachment include [1,2] interaction of an amphipathic α-helix that lies parallel to the membrane and imbeds in the outer leaflet [10,11], insertion of a hydrophobic loop or hydrophobic side chain into the hydrophobic core of the outer leaflet [6,9,12], insertion of one or more covalently bound lipid-like moieties into the hydrophobic core of the outer leaflet [3,4,13], and interaction through strong electrostatic or ionic interactions [14,15]. Permanent attachment is often achieved through combinations of these modes or through oligomerization. In contrast, peripheral membrane proteins are water-soluble proteins that bind reversibly with a lipid membrane. Reversible attachment is achieved through a combination of weak electrostatic and hydrophobic interactions [1,2,5,16,17]. In that case the interaction is well described by equilibrium binding constants [18–20].

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The classification described above defines the two limiting cases of protein–membrane association, but in reality there is a continuum of binding interaction strength. Often proteins bind rapidly, but dissociate slowly and only partially from the membrane on experimental timescales. Nevertheless, equilibrium relationships are commonly used to estimate an apparent binding constant (K_d) without establishing reversibility or rigorously justifying the validity of an equilibrium treatment [10,21–32]. The amount of protein that binds to a membrane is typically measured as a function of free protein concentration and the data are fit to an equilibrium equation to estimate a binding constant and free energy. However, if the system is not at equilibrium due to irreversible binding, the results will depend on the timescale or flow rate [33]. In some instances, K_d values from kinetic and steady state measurement methods are compared to verify reversibility [9,19,20,34,35], but that is generally not the case.

Evidence indicates that in many cases binding occurs in a two-stage process where an initial phase of rapid and reversible binding is followed by a transition to a second, more strongly bound state [2,10]. This scenario is illustrated by a conceptual free energy diagram shown in Fig. 1 for the soluble form of the Dengue virus envelope protein (sE) used in the present study. Several mechanisms may lead to this two-stage behavior. First, binding of a protein to a lipid membrane may involve an "induced fit", whereby protein residues and/or lipids rearrange to achieve the lowest free energy state [1,2]. While it is well known that some proteins undergo large conformational changes upon binding to lipid membranes, more subtle local segmental rearrangements likely take place even when large conformational changes do not occur. Other mechanisms resulting in two-stage protein–membrane interaction are equilibrium binding to the surface of a membrane followed by insertion [27,36] and protein–protein association after membrane binding [10,37,38]. In the case of Dengue sE, we argue below based on sedimentation analysis and also prior work that the protein most likely binds as monomers that subsequently associates to form trimers. Two-state models are sometimes used to describe such cases. If each state is reversible, then equilibrium relations still apply [36]. However, two-state equilibrium models are sometimes used as an approximation even when the second state is not reversible on experimental time scales [10].

Several methods are available to measure equilibrium binding of proteins to membranes. These include fluorescence methods, separation of membrane-bound protein by centrifugation, surface plasmon resonance, quartz crystal microbalance (QCM), and electron paramagnetic resonance spectroscopy. In contrast, few if any methods are available to probe the energetics of the release process for integral monotopic proteins or more generally, for proteins with unbinding free energies substantially greater than the thermal scale set by k_BT, where k_B is Boltzmann's constant and T is the absolute temperature. Atomic force microscopy (AFM) has been used previously to measure the force required to pull peptides out of membranes [39]. A number of disadvantages and difficulties with this method lessen its utility. Attaching the AFM tip to a single protein is challenging. In addition, due to the limited sensitivity of typical AFMs, the force can only be measured if it is greater than ~10 pN. Thus AFM is capable of measuring the force to pull transmembrane helices out of membranes, but proteins that are more weakly bound will be difficult or impossible to probe by this method. In addition, converting the measured force into an energy is challenging because the force depends upon the rate of retraction of the cantilever, and large forces may deform the protein or attachment point [40].

In summary, an understanding of the interactions between membranes and integral monotopic proteins, including amphitropic proteins, is lacking because current measurement techniques are limited to investigating reversible adsorption. Few, if any, methods are available to study proteins that are bound so strongly that dissociation is slow or negligible on experimental time scales. In those cases, it is unclear if functional outcomes are dependent on the rate of association or on strong, irreversible anchoring.

In this work we developed a new method to measure the activation energy (enthalpy ΔH^*_u and free energy ΔG^*_u) to remove strongly-bound membrane-associated proteins from a membrane. The method is based on coflotation of proteins bound to liposomes suspended in a sucrose gradient, where the rate of release of protein from the membrane is measured as a function of time and temperature upon spinning in an ultracentrifuge. Liposome coflotation is commonly used to assay for strong binding of proteins to lipid membranes [41]. We show below that an altered methodology can be used to determine ΔH^*_u and ΔG^*_u. Using this method we determined ΔH^*_u and ΔG^*_u of dengue sE inserted into membranes composed of 80:20 POPC:POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, a neutral lipid, and 1-palmitoyl-2-oleoyl-*sn*- glycero-3-phosphoglycerol, an anionic lipid). While prior structural studies of dengue sE were performed after binding and extracting sE from membranes of 1:1:1:3 POPC:POPE:sphingomyelin:cholesterol, we were motivated to examine membrane compositions involving negatively-charged lipids by a

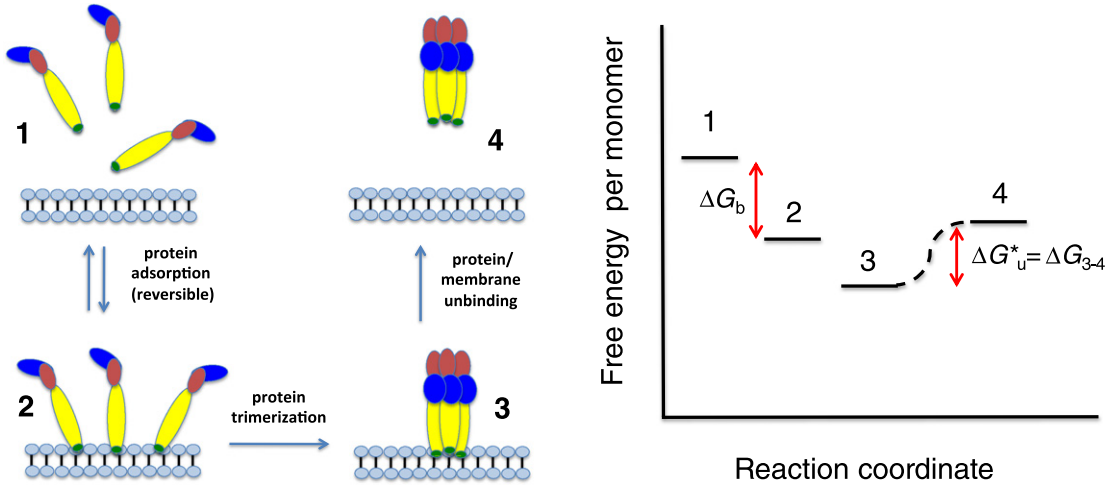

Fig. 1. a). Illustration of the binding, trimerization, and unbinding of Dengue sE with lipid membranes. b) Schematic free energy diagram for process illustrated in a). The red arrows indicate values measured in this work. The transition from 1 to 2 corresponds to association of a protein monomer with the membrane, measured here using a quartz crystal microbalance (QCM). Subsequent reorganization and trimer formation leads to state 3. Free energy per monomer for release of the trimer from the membrane is represented by the transition from 3 to 4, measured here in a coflotation/sedimentation assay. State 4 corresponds to a free trimer in solution.