Characterization of Membrane Protein Interactions by Isothermal Titration Calorimetry

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Abstract

Understanding the structure, folding, and interaction of membrane proteins requires experimental tools to quantify the association of transmembrane (TM) helices. Here, we introduce isothermal titration calorimetry (ITC) to measure integrin αIIbβ3 TM complex affinity, to study the consequences of helix–helix preorientation in lipid bilayers, and to examine protein-induced lipid reorganization. Phospholipid bicelles served as membrane mimics. The association of αIIbβ3 proceeded with a free energy change of −4.61 ± 0.04 kcal/mol at bicelle conditions where the sampling of random helix–helix orientations leads to complex formation. At bicelle conditions that approach a true bilayer structure in effect, an entropy saving of >1 kcal/mol was obtained from helix–helix preorientation. The magnitudes of enthalpy and entropy changes increased distinctly with bicelle dimensions, indicating long-range changes in bicelle lipid properties upon αIIbβ3 TM association. NMR spectroscopy confirmed ITC affinity measurements and revealed αIIbβ3 association and dissociation rates of 4500 ± 100 s^{-1} and 2.1 ± 0.1 s^{-1}, respectively. Thus, ITC is able to provide comprehensive insight into the interaction of membrane proteins.

Introduction

In typical genomes, 20–30% of genes encode membrane proteins [1] and many of which consist of single-pass transmembrane (TM) helices. The interaction between TM helices underlies critical signal transduction events, the assembly of membrane channels, and the association and folding of membrane proteins in general [2–6]. Deciphering the design principles of membrane proteins requires not only the structure determination of associated and dissociated TM helices but also the thermodynamic and kinetic understanding of TM helix–helix interactions. Likewise, an analogous characterization is required in order to understand the interaction of more complex membrane proteins. The interaction of TM helices *in vitro* has been quantified using approaches based on analytical ultracentrifugation, spectroscopic techniques, and trapping approaches [5,7–11]. A sedimentation equilibrium must be established and the reversibility of TM interactions has to be ensured, in order to conduct successful analytical ultracentrifugation [10]. Fluorescence spectroscopy can detect the Förster resonance energy transfer between a donor and an acceptor, which requires the tagging of TM peptides with fluorophores and the correction of the detected signal for the random colocalization of donor and acceptor [11]. NMR spectroscopy depends on the reliable detection of protein resonances [7,8], which limits applicable particle sizes to molecular masses of 30–50 kDa. Moreover, NMR is capable of determining the kinetic parameters of TM helix–helix association [12]. Trapping techniques require either the addition of assay-specific domains to the TM helices or disulfide formation between TM helices [5,9]. The present study introduces a new tool of measuring TM associations, isothermal titration calorimetry (ITC), which permits the label-free study of protein–protein interaction, provides a full set of thermodynamic parameter from a single experiment, and is not sensitive to particle size [13]. ITC has been used to study the interaction of membrane proteins with ligands in the aqueous phase [14–16], but to our knowledge, ITC has not been applied to the case

where both ligand and protein are in the hydrophobic phase. NMR is employed to validate ITC measurements and to determine TM helix–helix association and dissociation rate constants.

The homodimerization of TM helices has been extensively studied, for example, for glycophorin A and Erb receptors in micelles, bicelles, and unilamellar vesicles [7–9,17–19]. Analogous studies are necessary for the general case of helix–helix heterodimerization. To establish such a system, we characterize the thermodynamics and kinetics of integrin αIIbβ3 TM heterodimerization. Integrins are ubiquitous cell–cell and cell–matrix adhesion receptors [20] whose adhesive state is determined by the association state of the αβ TM helices [21]. Next to its model system character, an understanding of the αIIbβ3 TM complex has broad physiological and pathological significance [20,22].

When studying membrane proteins *in vitro*, it is generally not possible to preserve their native membrane environment and a membrane mimic has to be selected. Detergent micelles, phospholipid bicelles, nanodiscs, and unilamellar vesicles are common membrane substituents. TM peptides cannot transfer between individual nanodiscs or vesicles [11,23], which makes it necessary to premix interacting TM proteins. However, ITC depends on the direct titration of one peptide to its partnering peptide in the sample cells to detect relatively small heat changes upon complex formation. TM peptides may exchange between micelles and bicelles [7,12], which in principle allows ITC. The ensuing focus is on phospholipid bicelles, which exhibit a bilayer disc of long-chain lipids that is stabilized by a rim of short-chain lipids (Fig. 1). The radius of the bilayer disc is a function of the molar ratio of long-to-short chain lipids, the *q*-factor. The lipid environment offered by bicelles is critical to the structural integrity of the integrin αIIbβ3 TM complex [24,25].

TM helices have a preferred orientation relative to the plane of the membrane. In contrast, the isotropic tumbling of small bicelles in three dimensions removes any helix–helix orientation before association takes place. The entropy loss of TM complex formation in small bicelles is therefore greater than in membranes [26]. For the tetrameric M2 channel, a ΔG° difference of 0.7 kcal/mol between bilayers and micelles was measured normalized to each helix–helix interface [5]. Molecular dynamics simulations of glycophorin A suggest a penalty of ~1.3 kcal/mol for ΔG° in micelles compared to membranes [27]. Experimentally, structural differences were obtained for the glycophorin A structure in lipid vesicles and detergent micelles, which contributed to a relatively large $\Delta\Delta G^{\circ}$ of 4–5 kcal/mol [9]. It is therefore desirable to estimate the entropy differences between TM associations in two and three dimensions, termed $\Delta\Delta S^{\circ}_{3D}$, in the same membrane mimic. We utilize the scalability of the bicelle bilayer to attain TM helix–helix preorientation. Overall, it is shown that ITC is able to accurately measure the integrin αIIbβ3 TM complex affinity, to estimate the consequences of helix–helix preorientation, and to provide insight into protein-induced lipid reorganization.

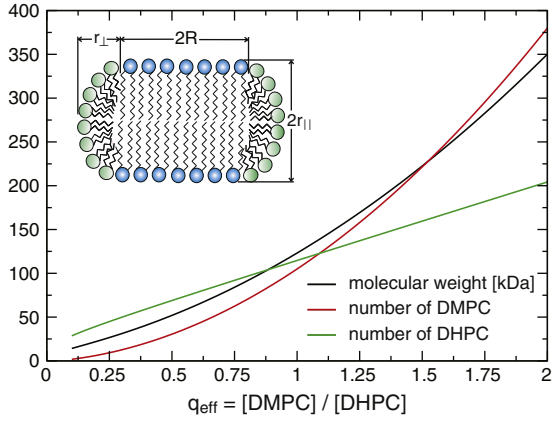

Fig. 1. Bicelle lipid aggregation number and molecular weight as a function of effective *q*-factor. The mixed micelle model was employed to calculate the depicted parameters for DHPC/DMPC bicelles using $2r_{||}$ = 40 Å, $r_{\perp}$ = 11 Å, ε = 0, ε′ = 0.03, V_{DMPC} = 1090 Å^3, and V_{DHPC} = 660 Å^3 [28]. The fraction of DHPC molecules inside the DMPC-rich section is ε while the fraction of DMPC molecules inside the DHPC-rich section is ε′. V_{DMPC} and V_{DHPC} denote the volumes of DMPC and DHPC molecules, respectively.

Results

Measurement of thermodynamic and kinetic constants by NMR

Throughout the present study, bicelles composed of 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine (DHPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were employed. Such bicelles are characterized extensively in terms of size and morphology [28–32]. The dependence of NMR linewidths on rotational correlation time limits NMR measurements of the integrin αIIbβ3 TM complex to the usage of bicelles with *q*-factors ≤0.5. Here, a *q*-factor of 0.34 was used to obtain spectra with high signal-to-noise ratios [24], whereby the concentration of free, non-bicellar DHPC of 7 mM [29] was excluded from *q*-factor calculations. To indicate this convention, we denote this effective *q*-factor as q_{eff}. The study of αIIbβ3 heterodimerization was facilitated by the documented absence of competing αIIb or β3 homodimerization [25,33,34]. At q_{eff} = 0.34, bicelles are relatively small (Fig. 1) and, at the employed lipid-to-peptide ratios, each TM peptide resides in one bicelle with many free, unoccupied bicelles remaining