Characterizing Membrane Proteins and Peptides by Calorimetry

Christin T. Choma
TA Instruments, 109 Lukens Drive, New Castle, DE 19720, USA

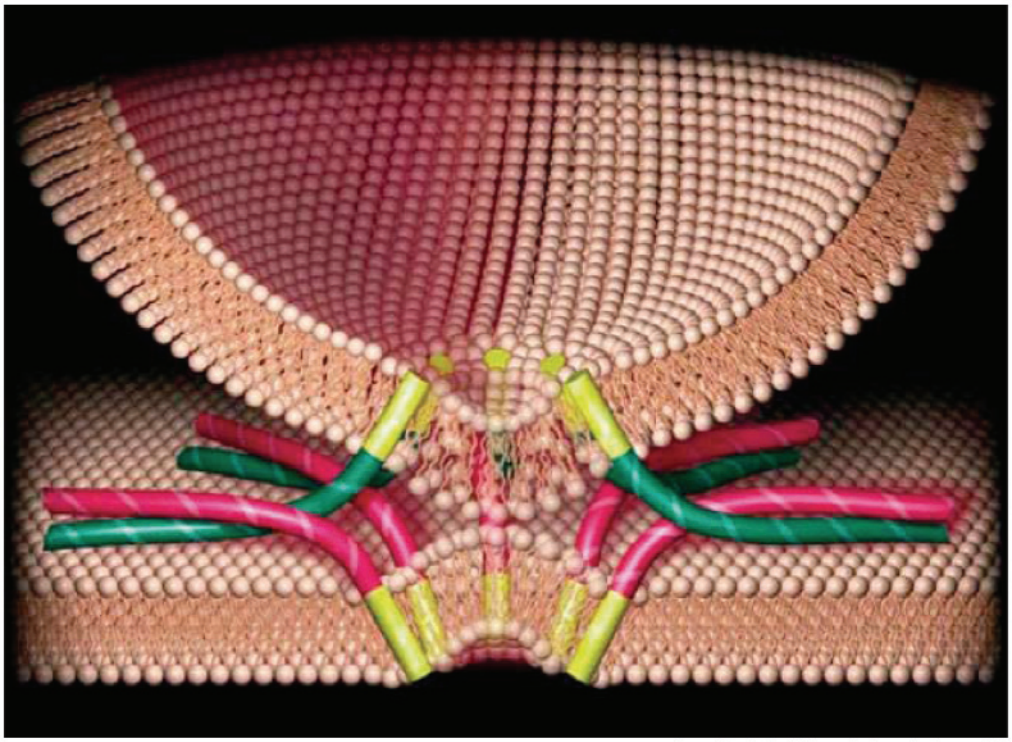

Illustration: © Craig Foster for James Rothman, PhD

Calorimetry is ideal for characterizing the stability of multi-component membrane protein/lipid assemblies such as the SNARE complex.

Although membrane proteins are very difficult to work with, ultra-sensitive calorimetry can overcome the limitations of many other physical characterization techniques to provide insights into the factors driving membrane protein/lipid assembly and controlling the stability of the complexes.

Proteins and peptides are integral components of all biological membranes. Embedded in the membrane or peripherally associated with the bilayer, membrane proteins and peptides have diverse biological functions ranging from receptors and ion channels to antibiotics and fungicides. Insights into the dynamics and interactions governing membrane protein and peptide functionality require an understanding of the molecular interactions between the lipid and polypeptide molecules (hydrophobic effects, hydrogen bonding and electrostatics), and the physical properties of the system, such as membrane fluidity and protein structure and stability. Since thermodynamics fundamentally govern these interactions and physical properties (Heerklotz, 2004), techniques which directly characterize the thermodynamic properties of membrane complexes provide a powerful approach for understanding the functionality of these systems. This application note explores the utility of differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) for characterizing the energetics governing membrane systems. For general descriptions of the principles behind DSC and ITC, please see Calorimetry Science Corporation's overview notes entitled *Life Science Applications of DSC* and *Life Science Applications of ITC*.

The Properties of Lipid Membranes

Lipids can form a variety of phase structures depending on their chemical structures, hydration state and temperature. When lipid/membrane protein systems are studied, lipids are generally fully hydrated and assembled in either unilamellar or multi-lamellar vesicles. DSC can directly monitor the effect of proteins and peptides on lipid phase structures by revealing both changes in the apparent molar heat capacity of the lipid, and changes in the temperature and sharpness of the melting transition. Pure lipids usually have very sharp, large enthalpy melting transitions which are broadened by association with a protein or peptide. The melting transition takes the membrane from a low-temperature gel phase, characterized by predominantly ordered acyl chains, to a high-temperature fluid phase in which the acyl chains are conformationally disordered. During this transition, the surface area of the membrane increases by about 25% and the membrane becomes thinner (Heimburg, 2000). In addition, saturated phosphatidylcholine membranes also exhibit a lower temperature, broad, low enthalpy pretransition in which the acyl chains go from fully ordered to predominantly ordered (with local point defects characterized by disordered acyl chains; Heimburg, 2000). The pretransition can be shifted 10 °C or more, or be completely abolished, by the addition of membrane proteins, peptides, or membrane-active drugs such as anesthetics (Fa *et al*., 2006).

Model membranes more closely approximating the properties of biological membranes are generally composed of several types of lipids, including cholesterol and sphingomyelin. In these more complex systems, groups of specific lipids may aggregate into patches or domains which have physical properties distinctly different from those of other lipid domains present elsewhere in the membrane. In addition, some lipids can exist either in a fluid or a gel phase at physiological temperature, resulting in distinct domains of a single lipid type that coexist in a membrane (Almeida *et al*., 2005). Most proteins and peptides interact with specific membrane domains through electrostatic attraction with the lipid headgroups, followed by partitioning into the bilayer, although if a peptide contains no charged groups, interaction is strictly by adsorption and partitioning. In either case, interaction with the bilayer often results in substantial conformational changes in peptides and, to a lesser extent, proteins (Seelig, 2004). The effect of bilayer structure on the structure and activity of membrane proteins (and the effect of integral membrane proteins on membrane structure) has been extensively reviewed (Lee, 2004).

Characterizing Vesicle Binding Using Isothermal Titration Calorimetry

Unlike site-specific binding (such as between a macromolecule and a ligand) the partitioning of a

peptide or protein into a membrane does not necessarily result in the formation of a specific complex with a defined stoichiometry. The thermodynamics and stoichiometry of membrane protein/lipid interactions are more akin to sorption isotherms than to site-specific binding. Nonetheless, the binding of peptides and proteins to membranes can be quickly and easily characterized by ITC, allowing determination of the binding constant (K_a) and reaction enthalpy. *Figure 1* illustrates a simple example, the binding of cyclosporin A to dipalmitoyl phosphatidylcholine (DPPC) vesicles. Cyclosporin A is a hydrophobic 11 residue cyclic peptide used clinically as an immunosuppressive agent. Since vesicles hold promise as potential drug carriers, the binding of hydrophobic drugs such as cyclosporin A to vesicles has relevance for clinical applications. The

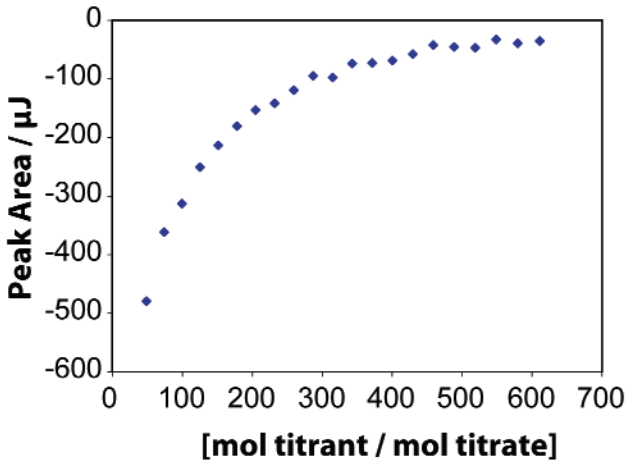

Figure 1. Cyclosporin A titrated with DPPC vesicles in a CSC model 5300 ITC-III. DPPC was sonicated in 1 mL water for 2 hr at 60 °C to provide a 70 mM suspension of small unilamellar vesicles. Cyclosporin A (1.8 mg), dissolved in 100 µL ethanol, was slowly added to 100 mL rapidly-stirred water (final concentration: 15 x 10^{-6} M cyclosporin A). The cyclosporin A solution was loaded into the 1.0 mL sample cell, and the DPPC suspension was loaded into a 250 µL syringe. Twenty-five, 10 µL aliquots of DPPC were titrated into the cyclosporin A solution while stirring at 200 rpm and maintaining the temperature of the system at 28 °C. Data were fit to an independent binding model; the graph shows the µJ from each injection plotted against the mole fraction of DPPC to cyclosporin A.

ITC data show that, on average, six DPPC molecules interact with each bound cyclosporin A, that the K_a of binding is approximately 390 M^{-1}, and that the enthalpy of binding is -61 kJ/mol DPPC.

In contrast to completely hydrophobic peptides like cyclosporin, peptides and proteins containing both charged and hydrophobic residues initially interact with vesicles via electrostatic interactions between their charged residues and the charged lipid headgroups, followed by at least partial penetration of their hydrophobic residues into the hydrophobic bilayer. ITC provides a straightforward approach for quantitatively characterizing this partitioning, as illustrated by the interaction of a cationic peptide, nisin Z, with membranes composed of different molar ratios of POPC and POPG (Breukink *et al.*, 2000). Nisin Z binding to neutral POPC vesicles yielded weak, but quantifiable, binding heats and a partition constant of approximately 540 M^{-1}. Although the peptide carries a +3.8 charge, the ITC binding isotherm could be best fit with a model assuming a charge of +1, suggesting that hydrophobic interactions dominate during binding and that the charged residues remain somewhat distant from the membrane. However, the addition of various amounts of negatively charged POPG resulted in substantially tighter binding and corresponding decreases in the intrinsic partition coefficient, indicating that the hydrophobic contribution to the binding process was much smaller compared to the case with neutral vesicles. By comparing the binding characteristics of several nisin Z mutants differing in their overall charge, the authors were able to separate electrostatic and hydrophobic contributions to the binding process, the partition constants, and the free energy and entropy of binding.

Many peptides undergo extensive conformational changes upon binding to a membrane, going from a random coil in solution to folded when bound. As with other physical techniques used to study conformational changes and binding of β-structure peptides to vesicles, the interpretation of ITC data of, for example, β-amyloid peptide binding to POPC/POPG vesicles, is

not straightforward. The analysis is complicated by the fact that the heat measured by the titration isotherm is comprised of conformational changes, a binding reaction, and an aggregation/dissociation reaction (Terzi *et al.*, 1995). In contrast, the investigation of α-helical peptide binding is relatively facile. For example, to study membrane-induced α-helix formation, the helical propensity of magainin (Weiprecht *et al.*, 1999) and a mitochondrial leader sequence peptide (Wieprecht *et al.*, 2000) were studied in POPC/POPG membranes. The helical propensity of magainin was systematically altered by substituting two adjacent amino acids with their D-enantiomers at various locations in the peptide. The substitution of residues near the center of the helix reduced helical content substantially more than substitutions near the termini. Using ITC, the binding enthalpies of the various magainin peptides were measured; when plotted as a function of helical content, an approximate straight line was obtained, allowing the binding enthalpy of an entirely random coil magainin peptide to be estimated from the y-axis intercept. The average contribution of a residue in the magainin sequence to the binding enthalpy (ΔH_{helix}) could then be calculated (-3.0 kJ/mol residue). Comparable experiments with a leader sequence peptide (Wieprecht *et al.*, 2000) provided a very similar value for ΔH_{helix}.

ITC binding studies with a given peptide or protein using either sonicated small unilamellar vesicles or extruded large unilamellar vesicles provide very similar binding constants and free energies, but significantly higher enthalpies of binding are observed with small vesicles, perhaps due to the different packing densities of small versus large vesicles (Wieprecht *et al.*, 2002; Seelig, 2004). It is also clear that amphipathic peptides bind exothermically to small unilamellar vesicles, and that enthalpy, rather than entropy, is the major driving force (Seelig and Ganz, 1991; Wieprecht *et al.*, 2000; Abraham *et al.*, 2005).

Characterizing Membrane Polypeptide Stability and Structure by Differential Scanning Calorimetry

DSC provides information on the thermal stability of membrane peptides and proteins, the domain and subunit structure of membrane proteins, and the effect that the interaction of a membrane protein or peptide has on the physical properties of the bilayer. The DSC analysis of membrane protein thermal stability and tertiary/quaternary structure is conducted in a manner analogous to that for water soluble proteins, and is described in detail in the CSC application notes entitled *Characterizing Protein Stability by DSC* and *Characterizing Protein Structure by DSC*. Specific examples of stability and structure studies on membrane peptides and proteins are reviewed in Shnyrov *et al.* (1997), Lee (2004) and Minetti and Remeta (2006).

DSC allows evaluation of the magnitude of intramolecular interactions (the unfolding enthalpy, ΔH) and the temperature-dependent exposure of polar and nonpolar residues to the solvent during unfolding (the change in heat capacity, ΔC_p). The principle differences observed between water soluble and membrane soluble proteins arise from the membrane-spanning portions of membrane proteins. Transmembrane α-helices and β-barrels tend to be very thermally stable and retain their secondary structure even after the extramembranous portions of the protein have unfolded. Consequently, ΔC_p is considerably lower for membrane proteins than for water soluble proteins because minimal hydrophobic surface area is exposed to the solvent upon unfolding. The unfolding enthalpy also tends to be lower than for water soluble proteins, indicating that the transmembrane segments of unfolded membrane proteins retain significant structure (Minetti and Remeta, 2006). Although solvent-exposed loops of membrane proteins unfold with enthalpies and heat capacities comparable to that observed for water soluble proteins, they nonetheless apparently contribute significantly to the stabilization of transmembrane α-helices and β-barrels (Minetti and Remeta, 2006).

Perhaps surprisingly, the overall stability of membrane proteins is comparable to that of water soluble proteins: approximately 4 KJ/mol per 10 Å of buried protein surface area (Faham *et al.*, 2004). Extensive mutational studies on bacteriorhodopsin indicate that

the free energy contribution arising from the burial of polar and non-polar residues within the bilayer is similar in membrane proteins (Faham *et al.*, 2004), whereas in water soluble proteins, the burial of non-polar residues is the major driving force for molten globule formation. Studies on self-oligomerizing model peptides indicate that the specific fold of a membrane protein is generally directed by hydrogen-bonded interactions between polar residues in the otherwise hydrophobic transmembrane sequence (DeGrado *et al.*, 2003). Although significantly less data exist for membrane proteins than for water soluble proteins, it appears that membrane proteins fold by packing preformed secondary structure units together, that packing is driven predominantly by van der Waals interactions, and that packing specificity is driven by specific hydrogen bonds between polar side chains buried in the lipid bilayer.

The effect of membrane proteins and other compounds on the ordered structure of a bilayer can be rapidly and sensitively studied by DSC. Saturated phospholipids such as DPPC undergo two principal thermally-induced transitions. The main phase transition gives rise to a high-enthalpy, narrow, highly co-operative peak due to the acyl chains transitioning from a highly ordered structure to a fluid phase in which the chains have substantial conformational disorder; the narrower this peak, the more co-operative the transition. This main transition is preceded by a low-enthalpy, broad pre-transition which corresponds to the melting or untilting of the of the lipid headgroups in the membrane. Both the pre- and main transitions are highly sensitive to perturbations in lipid packing, such as those caused by the binding and insertion of membrane proteins (Henzler-Wildman *et al.*, 2004). Generally, protein binding lowers transition temperatures, but protein interactions can also raise T_m (Huang *et al.*, 1997; Heimburg and Biltonen, 1996). For example, *Figure 2* shows the effect of two slightly different membrane protein complexes on the DSC profile of DPPC vesicles. Each 70 kDa complex is comprised of three recombinant, full-length SNARE proteins (transmembrane proteins which mediate the fusion of cellular vesicles with cell membranes). In one sample, one of the integral membrane proteins in the complex (SNAP-25) was chemically derivatized with palmitoyl groups, while the other two proteins in the complex, syntaxin (syx) and synaptobrevin (syb), were unmodified. This complex shifted both the pretransition and main transition of DPPC to higher temperatures, possibly due to favorable interactions of the palmitoyl groups with the lipid bilayer. Bilayer stabilization by palmitic acid has

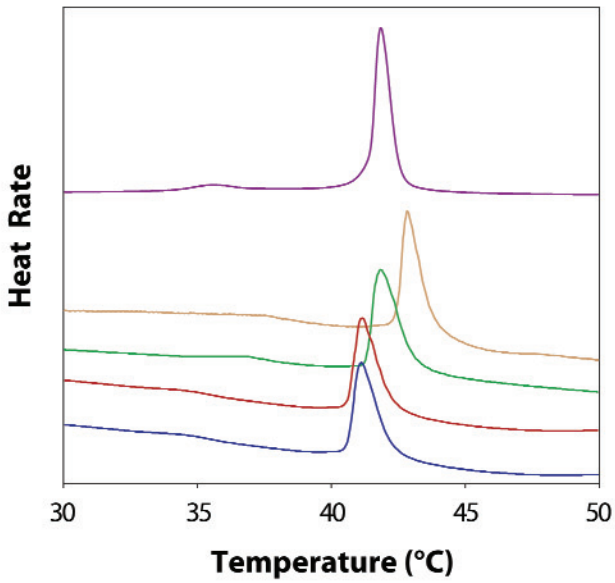

Figure 2. Effect of membrane proteins on the DSC profile of DPPC vesicles. Vesicles were prepared by bath sonication (5 mg/mL DPPC for 5 minutes) at room temperature in 150 mM KCl, 8 mM HEPES, pH 7. Degassed sample (DPPC alone or in the presence of protein) and buffer reference were loaded into an N-DSC III and scanned from 20-95 °C at 1 °C/min. Membrane proteins in the 300 µL sample cell were: palmitoylated complex (brown scan): 1.2 µg syx, 1.6 µg syb and 1.2 µg palmitoylated SNAP-25; non-palmitoylated complex (red scan): 1.2 µg syx, 1.6 µg syb and 1.2 µg non-palmitoylated SNAP-25; non-palmitoylated SNAP-25 (blue scan): 2 µg non-palmitoylated SNAP-25. The green scan corresponds to DPPC vesicles alone in buffer.

Customer requirements dictated that a relatively fast scan rate (1 °C/min) be used in the above experiment. However, compounds with very sharp transitions, such as lipids, are typically scanned more slowly to minimize peak broadening, as demonstrated by the scan of DPPC obtained at 0.5 °C/min (purple trace).

been previously reported (Henshaw *et al.*, 1998). In contrast, *Figure 2* also shows the DSC profile of DPPC vesicles in the presence of non-palmitylated SNAP-25 complex. In this case, the temperatures of the pre- and main transitions are both decreased, suggesting that the interaction of the protein complex with the membrane perturbs lipid packing. Furthermore, the interaction of non-palmitylated SNAP-25 alone (in the absence of syx and syb) essentially abolishes the pretransition and shifts the main transition to lower temperatures (*Figure 2*), consistent with the general observation that the interaction of membrane proteins with membranes has a destabilizing effect on the bilayer (Henzler-Wildman *et al.*, 2004).

Figure 3a

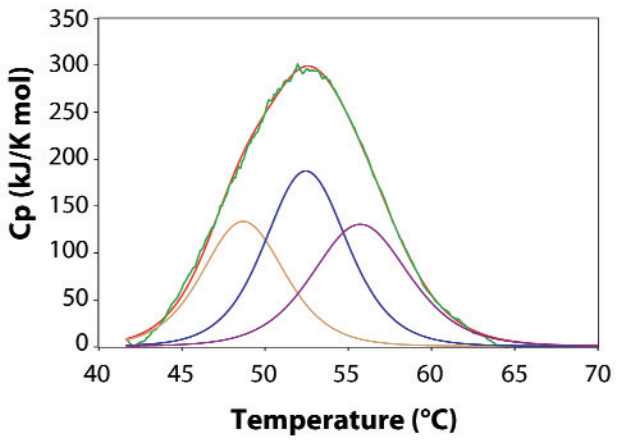

Figure 3b

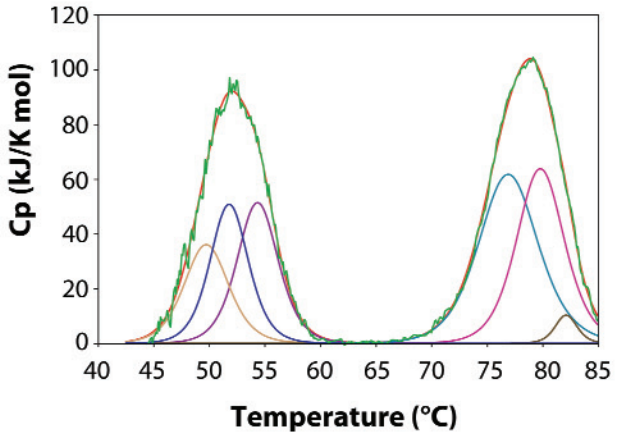

Figure 3. DSC scans of non-palmitoylated SNAP-25 complex (Figure 3a) and partially palmitoylated SNAP-25 complex (Figure 3b). Samples were prepared in 150 mM KCl, 8 mM HEPES, pH 7 buffer. Scans (green) were obtained from approximately 0.02 mg of complex in the 300 µL sample chamber, and were deconvoluted with the smallest number of transitions necessary to obtain a reasonable fit. (Data presented in Figures 2 and 3 were obtained in conjunction with D. Woodbury, Brigham Young University, Utah).

DSC can also provide information on the thermal stability of membrane proteins and protein complexes. For example, the non-palmitoylated SNARE complex mentioned above (syx, syb and SNAP-25) provided a DSC endotherm with a T_m of 52.8 °C that could be deconvoluted into three independent unfolding transitions (*Figure 3a*), consistent with the three-protein composition of the complex. The enthalpy of unfolding (2,300 kJ/mol, or approximately 33 J/g) is, on a per gram basis, very similar to that of lysozyme, a small, thermally stable protein. The relatively high enthalpy of the SNARE complex is consistent with its coiled coil structure. Partial palmitoylation of SNAP-25, followed by complex formation, showed two separate peaks on DSC (*Figure 3b*): one corresponding to the non-palmitoylated complex (T_m of 52.5 °C) and the second, with a T_m of 79.0 °C, corresponding to the thermally stabilized palmitoylated SNAP-25 complex. This higher temperature peak is consistent with melting temperatures reported for the native SNARE complex, which contains palmitoylated SNAP-25 (Fasshauer *et al.*, 2002).

Summary:

Membrane protein systems are notoriously difficult to work with: often only small quantities of sample are available at low concentrations, the preparations are rarely pure, and since the samples are usually suspensions, they are generally not optically clear. Ultra-sensitive calorimetry can often overcome these limitations and provide information not available by other techniques regarding the effects of peptides and proteins on membrane structure and stability (and vice versa), and on the dynamics of these interactions. As outlined in this application note and described in detail in the

references cited below, calorimetry provides the most direct approach for understanding the thermodynamics governing the stability of structures and the mechanisms by which membrane protein systems function.

References:

(Preference has been given to current references. Citation does not imply that a paper is necessarily the original reference to a study.)

Abraham, T., R. Lewis, R. S. Hodges and R. McElhaney (2005). Isothermal titration calorimetry studies of the binding of the antimicrobial peptide Gramicidin S to phospholipid bilayer membranes. *Biochemistry* **44**, 11279-11285.

Almeida, P. F. F., A. Pikorny and A. Hinderliter (2005). Thermodynamics of membrane domains. *Biochim. Biophys. Acta* **1720**, 1-13.

Breukink, E., P. Ganz, B. de Kruijff and J. Seelig (2000). Binding of nisin Z to bilayer vesicles as determined with isothermal titration calorimetry. *Biochemistry* **39**, 10247-10254.

DeGrado, W. F., H. Gratkowski and J. D. Lear (2003). How do helix-helix interactions help determine the folds of membrane proteins? Perspectives from the study of homo-oligomeric helical bundles. *Protein Science* **12**, 647-665.

Fa, N., A. Schanck, M. Deleu, A. Gaigneaux, E. Goormaghtigh and M. P. Mingeot-Leclercq (2006). Effect of the antibiotic azithromycin on the thermotropic behavior of DOPC or DPPC bilayers. *Chem. Phys. Lipids* **144**, 108-116.

Faham, S., D. Yang, E. Bare, S. Yohannan, J. P. Whitelegge and J. U. Bowie (2004). Side chain contributions to membrane protein structure and stability. *J. Mol. Biol.* **335**, 297-305.

Fasshauer, D., W. Antonin, V. Subramaniam and R. Jahn (2002). SNARE assembly and disassembly exhibit a pronounced hysteresis. *Nature Structural Biology* **9**, 144-151.

Heerklotz, H. (2004). The microcalorimetry of lipid membranes. *J. Phys.: Condens. Matter* **16**, R441-R467.

Heimburg, T. and R. L. Biltonen (1996). A Monte Carlo simulation study of protein-induced heat capacity changes and lipid-induced protein clustering. *Biophys J.* **70**, 84–96.

Heimburg, T. (2000). A model for the lipid pretransition: coupling of ripple formation with the chain-melting transition. *Biophys. J.* **78**, 1154-1165.

Henzler-Wildman, K. A., G. V. Martinez, M. F. Brown and A. Ramamoorthy (2004). Perturbation of the hydrophobic core of lipid bilayers by the humam antimicrobial peptide LL-37. *Biochemistry* **43**, 8459-8469.

Huang, W., L. P. Vernon, L. D. Hansen, and J. D. Bell (1997). Interactions of thionin from *Pyrularia pubera* with dipalmitoylphosphatidylglycerol large unilamellar vesicles. *Biochemistry* **36**, 2860 -2866.

Jolene B., J. B. Henshaw, C. A. Olsen, A. R. Farnbach, K. H. Nielson and J. D. Bell. (1998). Definition of the specific roles of lysolecithin and palmitic acid in altering the susceptibility of dipalmitoylphosphatidylcholine bilayers to phospholipase A2. *Biochemistry* **37**, 10709 -10721.

Lee, A. G. (2004). How lipids affect the activities of integral membrane proteins. *Biochim. Biophys. Acta* **1666**, 62-87.

Minetti, C. and D. P. Remeta (2006). Energetics of membrane protein folding and stability. *Archives Biochem. Biophys.* **453**, 32-53.

Seelig, J. and P. Ganz (1991). Nonclassical hydrophobic effect in membrane binding equilibria. *Biochemistry* **30**, 9354-9359.

Seelig, J. (2004). Thermodynamics of lipid-peptide interactions. *Biochim. Biophys. Acta* **1666,** 40-50.

Shnyrov, V. L., J. M. Sanchez-Ruiz, B. N. Boiko, G. G. Zhadan and E. A. Permyakov (1997). Applications of scanning microcalorimetry in biophysics and biochemistry. *Thermochem. Acta* **302**, 165-180.

Terzi, E., G. Holzemann and J. Seelig (1995). Self-association of beta-amyloid peptide (1-40) in solution and binding to lipid membranes. *J. Mol. Biol.* **252**, 633-642.

Wieprecht, T., O. Apostolov, M. Beyermann and J. Seelig (1999). Thermodynamics of the coil-alpha-helix transition of amphipathic peptides in a membrane

environment: implications for the peptide-membrane binding equilibrium. *J. Mol. Biol.* **294**, 785-794.

Wieprecht, T., O. Apostolov, M. Beyermann and J. Seelig (2000). Interaction of a mitochondrial presequence with lipid membranes: role of helix formation for membrane binding and perturbation. *Biochemistry* **39**, 15297-15305.

Wieprecht, T., M. Beyermann and J. Seelig (2002). Thermodynamics of the coil-alpha-helix transition of amphipathic peptides in a membrane environment: the role of vesicle curvature. *Biophys. Chem.* **96**, 191-201.