

DSC XXV –Pressure Perturbation Calorimetry

Barrett D. G., Minder C. M., Mian M. U., Whittington S. J., Cooper W. J., Fuchs K. M., Tripathy A., Waters M. L., Creamer T. P., and Pielak G. J. (2006) Pressure perturbation calorimetry of helical peptides. *Proteins* **63**, 322-326.

Abstract: Pressure perturbation calorimetry quantifies the temperature dependence of a solute's thermal expansion coefficient, providing information about solute-solvent interactions. We tested the idea that pressure perturbation calorimetry can provide information about solvent-accessible surface area by studying peptides with different secondary structures. The peptides comprised two host-guest series: one predominately an alpha-helix, the other predominately a polyproline II helix. In aqueous buffer, we find a correlation between the amount of secondary structure as assessed by circular dichroism spectropolarimetry and the pressure perturbation calorimetry data. We conclude that pressure perturbation calorimetry can provide information about the exposure of polar and nonpolar surface area. Data acquired in a buffered urea solution, however, are not as easily interpreted.

Batchelor J. D., Olteanu A., Tripathy A., and Pielak G. J. (2004) Impact of protein denaturants and stabilizers on water structure. *J Am Chem Soc* **126**, 1958-1961.

Abstract: It is of great interest to determine how solutes such as urea, sugars, guanidinium salts, and trimethylamine N-oxide affect the stability, solubility, and solvation of globular proteins. A key hypothesis in this field states that solutes affect protein stability indirectly by making or breaking water structure. We used a new technique, pressure perturbation calorimetry, to measure the temperature dependence of a solute's partial compressibility. Using fundamental thermodynamic relations, we converted these data to the pressure dependence of the partial heat capacity to examine the impact of protein stabilizing and denaturing solutes on water structure by applying the classic two-state mixture model for water. Contrary to widely held expectations, we found no correlation between a solute's impact on water structure and its effect on protein stability. Our results indicate that efforts to explain solute effects should focus on other hypotheses, including those based on preferential interaction and excluded volume.

Boehm K., Guddorf J., and Hinz H. J. (2007) Application of pressure-modulated differential scanning calorimetry to the determination of relaxation kinetics of multilamellar lipid vesicles. *Biophys Chem* **126**, 4241-4249.

Abstract: We report an extension of the recently published PMDSC method that permitted synchronous determination of heat capacity and expansibility when using slow, defined pressure formats in a DSC scan. Here we applied continuously opposing pressure changes that are fast compared to the time constants of the DSC instrument to study relaxation kinetics of phospholipids. Investigations of multilamellar vesicles of DPPC or DSPC in water revealed for both lipids relaxation times of about 30 s at the maximum of the main transition peak and about 15 s at the maximum of the pretransition. The relaxation times in the transition range are proportional to heat capacity of main- and pretransition. The molecular origin of the relaxation processes appears to stem from pressure-induced water fluxes between the interbilayer region and the bulk water phase.

Boehm K., Rosgen J., and Hinz H. J. (2006) Pressure-modulated differential scanning calorimetry. An approach to the continuous, simultaneous determination of heat capacities and expansion coefficients. *Anal Chem* **78**, 984-990.

Abstract: A new method is described that permits the continuous and synchronous determination of heat capacity and expansibility data. We refer to it as pressure-modulated differential scanning calorimetry (PMDSC), as it involves a standard DSC temperature scan and superimposes on it a pressure modulation of preselected format. The power of the method is demonstrated using salt solutions for which the most accurate heat capacity and expansibility data exist in the literature. As the PMDSC measurements could reproduce the parameters with high accuracy and precision, we applied the method also to an aqueous suspension of multilamellar DSPC vesicles for which no expansibility data had been reported previously for the transition region. Excellent agreement was obtained between data from PMDSC and values from independent direct differential scanning densimetry measurements. The basic theoretical background of the method when using sawtooth-like pressure ramps is given under Supporting Information, and a complete statistical thermodynamic derivation of the general equations is presented in the accompanying paper.

Chong P. L., Ravindra R., Khurana M., English V., and Winter R. (2005) Pressure Perturbation and Differential Scanning Calorimetric Studies of Bipolar Tetraether Liposomes Derived from the Thermoacidophilic Archaeon *Sulfolobus Acidocaldarius*. *Biophys J* **89**, 1841-1849.

Abstract: Differential scanning (DSC) and pressure perturbation calorimetry (PPC) were used to characterize thermal phase transitions, membrane packing, and volumetric properties in multilamellar vesicles (MLVs) composed of the polar lipid fraction E (PLFE) isolated from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* grown at different temperatures. For PLFE MLVs derived from cells grown at 78°C, the first DSC heating scan exhibits an endothermic transition at 46.7°C, a small hump near 60°C, and a broad exothermic transition at 78.5°C, whereas the PPC scan reveals two transitions at ~45 and 60°C. The endothermic peak at 47°C is attributed to a lamellar-to-lamellar phase transition and has an unusually low ΔH (3.5 kJ/mol) and $\Delta V/V$ (0.1%) value, as compared to those for the main phase transitions of saturated diacyl monopolar diester lipids. This result may arise from the restricted trans-gauche conformational changes in the dibiphytanyl chain due to the presence of cyclopentane rings and branched methyl groups and due to the spanning of the lipid molecules over the whole membrane. The exothermic peak at 78.5°C probably corresponds to a lamellar-to-cubic phase transition and exhibits a large and negative ΔH value (-23.2 kJ/mol), which is uncommon for normal lamellar-to-cubic phospholipid phase transformations. This exothermic transition disappears in the subsequent heating scans and thus may involve a metastable phase, which is irreversible at the scan rate used. Further, there is no distinct peak in the plot of the thermal expansion coefficient α vs. temperature near 78.5°C, indicating that this lamellar-to-cubic phase transition is not accompanied by any significant volume change. For PLFE MLVs derived from cells grown at 65°C, similar DSC and PPC profiles and thermal history responses were obtained. However, the lower growth temperature yields a higher $\Delta V/V$ (~0.25%) and ΔH (14 kJ/mol) value for the lamellar-to-lamellar phase transition measured at the same pH (2.1). A lower growth temperature also generates a less negative temperature dependence of α . The changes in $\Delta V/V$, ΔH , and the temperature dependence of α can be attributed to the decrease in the number of cyclopentane rings in PLFE at the lower growth temperature. The relatively low $\Delta V/V$ and small ΔH involved in the phase transitions help to explain why PLFE liposomes are remarkably thermally stable and also echo the proposal that PLFE liposomes are generally rigid and tightly packed. These results help us to understand why, in spite of the occurrence of thermal-induced phase transitions, PLFE liposomes exhibit a remarkably low temperature sensitivity of proton permeation and dye leakage.

Cooper A., Johnson C. M., Lakey J. H., and Nollmann M. (2001) Heat does not come in different colours: entropy-enthalpy compensation, free energy windows, quantum confinement, pressure perturbation calorimetry, solvation and the multiple causes of heat capacity effects in biomolecular interactions. *Biophys Chem* **93**, 215-230.

Abstract: Modern techniques in microcalorimetry allow us to measure directly the heat changes and associated thermodynamics for biomolecular processes in aqueous solution at reasonable concentrations. All these processes involve changes in solvation/hydration, and it is natural to assume that the heats for these processes should reflect, in some way, such changes in solvation. However, the interpretation of data is still somewhat ambiguous, since different non-covalent interactions may have similar thermodynamic signatures, and analysis is frustrated by large entropy-enthalpy compensation effects. Changes in heat capacity (ΔC_p) have been related to changes in hydrophobic hydration and non-polar accessible surface areas, but more recent empirical and theoretical work has shown how this need not always be the case. Entropy-enthalpy compensation is a natural consequence of finite ΔC_p values and, more generally, can arise as a result of quantum confinement effects, multiple weak interactions, and limited free energy windows, giving rise to thermodynamic homeostasis that may be of evolutionary and functional advantage. The new technique of pressure perturbation calorimetry (PPC) has enormous potential here as a means of probing solvation-related volumetric changes in biomolecules at modest pressures, as illustrated with preliminary data for a simple protein-inhibitor complex.

Cooper A., Cameron D., Jakus J. and Pettigrew G. W. (2007) Pressure perturbation calorimetry, heat capacity and the role of water in protein stability and interactions. *Biochem Soc. Trans.* **35**, 1547-1550.

Abstract: It is widely acknowledged, and usually self-evident, that solvent water plays a crucial role in the overall thermodynamics of protein stabilization and biomolecular interactions. Yet we lack experimental techniques that can probe unambiguously the nature of protein-water or ligand-water interactions and how they might change during protein folding or ligand binding. PPC (pressure perturbation calorimetry) is a

relatively new technique based on detection of the heat effects arising from application of relatively small pressure perturbations (+/-5 atm; 1 atm=101.325 kPa) to dilute aqueous solutions of proteins or other biomolecules. We show here how this can be related to changes in solvation/hydration during protein-protein and protein-ligand interactions. Measurements of 'anomalous' heat capacity effects in a wide variety of biomolecular interactions can also be related to solvation effects as part of a quite fundamental principle that is emerging, showing how the apparently unusual thermodynamics of interactions in water can be rationalized as an inevitable consequence of processes involving the co-operative interaction of multiple weak interactions. This leads to a generic picture of the thermodynamics of protein folding stabilization in which hydrogen-bonding plays a much more prominent role than has been hitherto supposed.

Cordeiro Y., Kraineva J., Ravindra R., Lima L. M., Gomes M. P., Foguel D., Winter R., and Silva J. L. (2004) Hydration and packing effects on prion folding and beta-sheet conversion. High pressure spectroscopy and pressure perturbation calorimetry studies. *J Biol Chem* **279**, 32354-32359.

Abstract: The main hypothesis for prion diseases proposes that the cellular protein (PrP(C)) can be altered into a misfolded, beta-sheet-rich isoform (PrP(Sc)), which undergoes aggregation and triggers the onset of transmissible spongiform encephalopathies. Here, we compare the stability against pressure and the thermomechanical properties of the alpha-helical and beta-sheet conformations of recombinant murine prion protein, designated as alpha-rPrP and beta-rPrP, respectively. High temperature induces aggregates and a large gain in intermolecular antiparallel beta-sheet (beta-rPrP), a conformation that shares structural similarity with PrP(Sc). alpha-rPrP is highly stable, and only pressures above 5 kilobars (1 kilobar = 100 MegaPascals) cause reversible denaturation, a process that leads to a random and turn-rich conformation with concomitant loss of alpha-helix, as measured by Fourier transform infrared spectroscopy. In contrast, aggregates of beta-rPrP are very sensitive to pressure, undergoing transition into a dissociated species that differs from the denatured form derived from alpha-rPrP. The higher susceptibility to pressure of beta-rPrP can be explained by its less hydrated structure. Pressure perturbation calorimetry supports the view that the accessible surface area of alpha-rPrP is much higher than that of beta-rPrP, which explains the lower degree of hydration of beta-rPrP. Our findings shed new light on the mechanism of prion conversion and show how water plays a prominent role. Our results allow us to propose a volume and free energy diagram of the different species involved in the conversion and aggregation. The existence of different folded conformations as well as different denatured states of PrP may explain the elusive character of its conversion into a pathogenic form.

Cordeiro Y., Kraineva J., Winter R., and Silva J.L. (2005) Volume and energy folding landscape of prion protein revealed by pressure. *Braz J Med Biol Res.* **38**, 1195-201.

Abstract: The main hypothesis for prion diseases proposes that the cellular protein (PrP C) can be altered into a misfolded, ss-sheet-rich isoform, the PrP Sc (from scrapie). The formation of this abnormal isoform then triggers the transmissible spongiform encephalopathies. Here, we discuss the use of high pressure as a tool to investigate this structural transition and to populate possible intermediates in the folding/unfolding pathway of the prion protein. The latest findings on the application of high pressure to the cellular prion protein and to the scrapie PrP forms will be summarized in this review, which focuses on the energetic and volumetric properties of prion folding and conversion.

Dzwolak W., Grudzielanek S., Smirnovas V., Ravindra R., Nicolini C., Jansen R., Lokszejn A., Porowski S., and Winter R. (2005) Ethanol-perturbed amyloidogenic self-assembly of insulin: looking for origins of amyloid strains. *Biochemistry* **44**, 8948-8958.

Abstract: A model cosolvent, ethanol, has profound and diversified effects on the amyloidogenic self-assembly of insulin, yielding spectroscopically and morphologically distinguishable forms of beta-aggregates. The alcohol reduces hydrodynamic radii of insulin molecules, decreases enthalpic costs associated with aggregation-prone intermediate states, and accelerates the aggregation itself. Increasing the concentration of the cosolvent promotes curved, amorphous, and finally donut-shaped forms. According to FT-IR data, inter-beta-strand hydrogen bonding is stronger in fibrils formed in the presence of ethanol. Mechanisms underlying the polymorphism of insulin aggregates were investigated by spectroscopic (CD, FT-IR, and fluorescence anisotropy) and calorimetric (DSC and PPC) methods. The nonmonotonic character of the influence of ethanol on insulin aggregation suggests that both preferential exclusion (predominant at the low concentrations) and direct alcohol-protein interactions are involved. The perturbed hydration of aggregation nuclei appears to be a decisive factor in selection of a dominant mode of beta-

strand alignment. It may override unfavorable structural consequences of an alternative strand-to-strand stacking, such as strained hydrogen bonding. A hypothetical mechanism of inducing different amyloid "strains" has been put forward. The cooperative character of fibril assembly creates enormous energy barriers for any interstrain transition, which renders the energy landscape comblike-shaped.

Dzwolak W., Ravindra R., Lendermann J., and Winter R. (2003) Aggregation of bovine insulin probed by DSC/PPC calorimetry and FTIR spectroscopy. *Biochemistry* **42**, 11347-11355.

Abstract: Pressure perturbation calorimetry (PPC), differential scanning calorimetry (DSC), and time-resolved Fourier transform infrared spectroscopy (FTIR) have been employed to investigate aggregation of bovine insulin at pH 1.9. The aggregation process exhibits two distinguished phases. In the first phase, an intermediate molten globule-like conformational state is transiently formed, reflected by loose tertiary contacts and a robust H/D-exchange. This is followed by unfolding of the native secondary structure. The unfolding of insulin is fast, endothermic, partly reversible, and accompanied by a volume expansion of approximately 0.2%. The second phase consists of actual aggregation: an exothermic irreversible process revealing typical features of nucleation-controlled kinetics. The volumetric changes associated with the second phase are small. The concentration-dependence of DSC scans does not support a monomer intermediate model. While insulin aggregation under ambient pressure is fast and quantitative, pressure as low as 300 bar is sufficient to prevent the aggregation completely, as high-pressure FTIR spectroscopy revealed. This is explained in terms of the high pressure having an adverse effect on the thermal unfolding of insulin, and therefore preventing occurrence of the aggregation-prone intermediate. A comparison of the aggregation in H(2)O and D(2)O shows that the isotopic substitution has diverse effects on both the phases of aggregation. In heavy water, a more pronounced volume expansion accompanies the unfolding stage, while only the second phase shifts to higher temperature.

Dzwolak W., Ravindra R., Nicolini C., Jansen R., and Winter R. (2004) The diastereomeric assembly of polylysine is the low-volume pathway for preferential formation of beta-sheet aggregates. *J Am Chem Soc* **126**, 3762-3768.

Abstract: The interaction of left- and right-handed polylysine chains (poly(D-lysine) and poly(L-lysine)) results in a dramatic increase in the propensity to form aggregated beta-sheet structure (and amyloid-like fibrils), which is reflected by an approximately 15 degrees C decrease of temperature of the alpha-helix-to-beta-sheet transition. While a relative volume expansion of 13-19 mL x mol⁻¹ accompanies the alpha-to-beta-transition in a single enantiomer, this does not hold true for the mixture, which, along with substantially more negative heat capacity changes, points to a lower solvent-entropy cost of the transition as the possible thermodynamic driving force of the diastereomeric aggregation. The underlying solvational mechanism may be one of the decisive factors responsible for the spontaneous protein aggregation in vivo and, as such, may shed new light on the molecular basis of amyloid-associated diseases.

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

Abstract: Insight into the forces governing a system is essential for understanding its behaviour and function. Calorimetric investigations provide a wealth of information that is not, or is hardly, available by other methods. This paper reviews calorimetric approaches and assays for the study of lipid vesicles (liposomes) and biological membranes. With respect to the instrumentation, differential scanning calorimetry (DSC), pressure perturbation calorimetry (PPC), isothermal titration calorimetry (ITC) and water sorption calorimetry are considered. Applications of these techniques to lipid systems include the measurement of thermodynamic parameters and a detailed characterization of the thermotropic, barotropic, and lyotropic phase behaviour. The membrane binding or partitioning of solutes (proteins, peptides, drugs, surfactants, ions, etc) can also be quantified. Many calorimetric assays are available for studying the effect of proteins and other additives on membranes, characterizing non-ideal mixing, domain formation, stability, curvature strain, permeability, solubilization, and fusion. Studies of membrane proteins in lipid environments elucidate lipid-protein interactions in membranes. The systems are described in terms of enthalpic and entropic forces, equilibrium constants, heat capacities, partial volume changes etc, shedding light also on the stability of structures and the molecular origin and mechanism of structural changes.

Heerklotz H., Tsamaloukas A., Kita-Tokarczyk K., Strunz P., and Gutberlet T. (2004) Structural, Volumetric, and Thermodynamic Characterization of a Micellar Sphere-to-Rod Transition. *J Am Chem Soc*

126, 16544-16552.

Abstract: The thermotropic sphere-to-rod transition of nonionic surfactants was characterized in terms of a large set of parameters: the transition temperature and width, the partial volume, coefficient of thermal volume expansion, enthalpy, isobaric heat capacity, and structural parameters, such as radius of gyration and hydrodynamic radius. Data were recorded as a function of concentration of surfactants in H₂O and in D₂O. To this end, pressure perturbation calorimetry (PPC), small angle neutron scattering (SANS), dynamic light scattering (DLS), differential scanning calorimetry (DSC), and isothermal titration calorimetry (ITC) were applied in a study of aqueous solutions containing myristyl, tridecyl, and lauryl maltoside and heptaethyleneglycoltetradecyl ether (C(14)EO(7)). Small changes in the thermodynamic and volumetric parameters (e.g., the partial volume change is approximately +2 per thousand) are discussed in detail as the result of three effects governing the transition. (i) Reduction of the water accessible hydrophobic surface area (ASA_{ap}) drives the transition. (ii) Shrinking in headgroup size by thermal dehydration triggers the transition. (iii) Hypothesized gradual ordering of the chains may control the effect of chain length on the transition.

Heerklotz H. and Seelig J. (2002) Application of pressure perturbation calorimetry to lipid bilayers. *Biophys J* **82**, 1445-1452.

Abstract: Pressure perturbation calorimetry (PPC) is a new method that measures the heat consumed or released by a sample after a sudden pressure jump. The heat change can be used to derive the thermal volume expansion coefficient, $\alpha(V)$, as a function of temperature and, in the case of phase transitions, the volume change, ΔV , occurring at the phase transition. Here we present the first report on the application of PPC to determine these quantities for lipid bilayers. We measure the volume changes of the pretransition and main transition of 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and the thermal expansivity of the fluid phase of DMPC and of two unsaturated lipids, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine and 1,2-dioleoyl-sn-glycero-3-phosphocholine. The high sensitivity of PPC instrumentation gives accurate data for $\alpha(V)$ and ΔV even upon the application of relatively low pressures of approximately 5 bar.

Heerklotz H. (2002) Triton promotes domain formation in lipid raft mixtures. *Biophys J* **83**, 2693-2701.

Abstract: Biological membranes are supposed to contain functional domains (lipid rafts) made up in particular of sphingomyelin and cholesterol, glycolipids, and certain proteins. It is often assumed that the application of the detergent Triton at 4 degrees C allows the isolation of these rafts as a detergent-resistant membrane fraction. The current study aims to clarify whether and how Triton changes the domain properties. To this end, temperature-dependent transitions in vesicles of an equimolar mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, egg sphingomyelin, and cholesterol were monitored at different Triton concentrations by differential scanning calorimetry and pressure perturbation calorimetry. Transitions initiated by the addition of Triton to the lipid mixture were studied by isothermal titration calorimetry, and the structure was investigated by ³¹P-NMR. The results are discussed in terms of liquid-disordered (ld) and -ordered (lo) bilayer and micellar (mic) phases, and the typical sequence encountered with increasing Triton content or decreasing temperature is ld, ld + lo, ld + lo + mic, and lo + mic. That means that addition of Triton may create ordered domains in a homogeneous fluid membrane, which are, in turn, Triton resistant upon subsequent membrane solubilization. Hence, detergent-resistant membranes should not be assumed to resemble biological rafts in size, structure, composition, or even existence. Functional rafts may not be steady phenomena; they might form, grow, cluster or break up, shrink, and vanish according to functional requirements, regulated by rather subtle changes in the activity of membrane disordering or ordering compounds.

Heerklotz H. and Tsamaloukas A. (2006) Gradual change or phase transition: characterizing fluid lipid-cholesterol membranes on the basis of thermal volume changes. *Biophys J* **91**, 600-607.

Abstract: Cholesterol has been reported to govern biomembrane permeability, elasticity, and the formation of lipid rafts. There has been a controversy whether binary lipid-cholesterol membranes should better be described in terms of a phase separation (liquid-ordered and liquid-disordered phases) or of gradual changes in largely homogeneous membranes. We present a new approach for detecting and characterizing phase equilibria in colloidal dispersions using pressure perturbation calorimetry (PPC). We apply this to the study of the thermal expansivity of mixtures of 1-palmitoyl-2-oleoyl sn-glycero-3-phosphatidylcholine (POPC) and cholesterol as a function of composition and temperature. We show that cholesterol can

condense lipids not only laterally (with respect to interfacial area) but also in volume. A quantitative comparison with expansivity curves simulated assuming either phase separation or random mixing within one phase reveals that the real system shows an intermediate behavior due to submicroscopic demixing effects. However, both models yield consistent system parameters and are thus found to be useful for describing the systems to a similar approximation. Accordingly, one cholesterol may condense 3 +/- 1 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine molecules by approximately -(1.4 +/- 0.5) vol % at 2 degrees C; both absolute values decrease with increasing temperature.

Heerklotz P. D. (2007) Pressure perturbation calorimetry. *Methods Mol Biol* **400**, 197-206.

Abstract: Pressure perturbation calorimetry is a rather new technique which serves to measure the temperature-dependent thermal volume expansion of a solute or particle in aqueous dispersion. It can be used to detect thermotropic transitions in lipid systems and to characterize their accompanying volume changes and kinetics. The results are of highest precision and obtained in a very convenient, fully automated experiment, requiring relatively little material. The strategy of the technique is to measure the heat response to a very little, isothermal pressure perturbation in a high-sensitivity isothermal calorimeter. On the basis of such data, thermodynamic laws and considerations yield the thermal expansion of the partial volume of the solute or colloidal particle.

Herberhold H., Royer C. A., and Winter R. (2004) Effects of chaotropic and kosmotropic cosolvents on the pressure-induced unfolding and denaturation of proteins: an FT-IR study on staphylococcal nuclease. *Biochemistry* **43**, 3336-3345.

Abstract: FT-IR spectroscopy was used to study the effects of various chaotropic and kosmotropic cosolvents (glycerol, sucrose, sorbitol, K₂SO₄, CaCl₂, and urea) on the secondary structure and thermodynamic properties upon unfolding and denaturation of staphylococcal nuclease (Snase). The data show that the different cosolvents have a profound effect on the denaturation pressure and the Gibbs free energy (ΔG°) and volume (ΔV°) change of unfolding. Moreover, by analysis of the amide I' infrared bands, conformational changes of the protein upon unfolding in the different cosolvents have been determined. An increase, a reduction, or an independence of the volume change of unfolding is observed, depending on the type of cosolvent, which can at least in part be attributed to the formation of a different unfolded state structure of the protein. The data are compared with the corresponding thermodynamic values of ΔV° for the temperature-induced unfolding process of Snase as obtained by pressure perturbation calorimetry, and significant differences are observed and discussed.

Kamerzell T. J., Ramsey J. D. and Middaugh C. R. (2008) Immunoglobulin dynamics, conformational fluctuations, and nonlinear elasticity and their effects on stability. *J Phys. Chem B* **112**, 3240-3250.

Abstract: The relationships between protein dynamics, function, and stability are incompletely understood. Two external perturbations (temperature and pH) were used to modulate the flexibility and stability of an IgG1kappa monoclonal antibody (mAb) in an attempt to better understand the possible correlations between flexibility and stability. Ultrasonic velocimetry, densitometry, differential scanning calorimetry (DSC), and pressure perturbation calorimetry (PPC) were used to experimentally determine the adiabatic and isothermal compressibility, expansibility, fractional volumes of unfolding, and various nonlinear thermoacoustical parameters as a function of pH and temperature. By combining these results, state parameter fluctuations were calculated from fundamental statistical mechanical relationships. The most dynamic and rigid mAb ensemble is measured at pH 4 and 6, respectively, based on state parameter fluctuations and compressibility. The effect of pH appears to couple mAb dynamics to solvent fluctuations, which control its dynamics and stability. A nonlinear response to mechanical perturbation, comparable to that seen with many polymers, is observed for this monoclonal antibody at pH 4-8. This behavior is characterized as strongly anisotropic and anharmonic, especially at pH 4. The midpoint of thermal unfolding as measured by DSC does not necessarily correlate with flexibility

Krivanek R., Okoro L. and Winter R. (2008) Effect of cholesterol and ergosterol on the compressibility and volume fluctuations of phospholipid-sterol bilayers in the critical point region: a molecular acoustic and calorimetric study. *Biophys J* **94**, 3538-3548.

Abstract: Although sterol-phospholipid interactions have been of interest for many years now, a complete thermodynamic profile of these systems is still missing. To contribute to a better understanding of the thermodynamic functions of these systems, we determined isothermal compressibility coefficient data for

dipalmitoylphosphocholine (DPPC) and DPPC-containing cholesterol and ergosterol vesicles by means of molecular acoustics (ultrasound velocimetry and densimetry) and differential scanning and pressure perturbation calorimetric techniques. A particular focus was on the influence of the differential structural properties of the two sterols on the thermodynamic properties of lipid bilayers, and on the nature of the critical point region of phospholipid-sterol systems by determining thermodynamic fluctuation parameters. Contrary to significant changes in conformational and dynamical properties of the DPPC-sterol membranes, no marked differences were found in the various thermodynamic properties studied, including the adiabatic ($\beta(S)(\text{lipid})$) and isothermal ($\beta(T)(\text{lipid})$) compressibility, as well as the volume fluctuations. Differences in $\beta(T)(\text{lipid})$ and $\beta(S)(\text{lipid})$ become dramatic in the gel-fluid transition region only, due to a significant degree of slow relaxational processes in the microsecond time range in the transition region. Our data show no evidence for the existence of a typical critical point phenomenon in the concentration and temperature range where a critical point in the DPPC-sterol phase diagram is expected to appear. Hence, on a macroscopic level, it seems more appropriate to describe the sterol-phospholipid binary mixtures in the liquid-ordered/liquid-disordered coexistence region as a phase region consisting essentially of small nanodomains only. Such small-domain dimensions, with a series of particular properties such as increased line energy, spontaneous curvature, and limited lifetime, seem also to be typical of raftlike domains in cell membranes

Kujawa P, and Winnik FM. (2001) Volumetric Studies of Aqueous Polymer Solutions Using Pressure Perturbation Calorimetry: A New Look at the Temperature-Induced Phase Transition of Poly(*N*-isopropylacrylamide) in Water and D₂O. *Macromolecules* **34**, 4130–4135.

Abstract: We report the first application of pressure perturbation calorimetry (PPC) to determine the hydration properties of poly(*N*-isopropylacrylamide) (PNIPAM) in H₂O and in D₂O as the solutions undergo a temperature-induced phase transition. The technique, which measures the heat change resulting from a pressure change above a solution of PNIPAM placed in a microcalorimeter cell, yields the temperature dependence of the coefficient of thermal expansion, α_p , of the polymer in solution and the change in volume of the solvation layer around the polymer chain. In the temperature ranges below and above the phase transition, α_p of PNIPAM in H₂O increased linearly with temperature. It underwent a sharp increase at the transition temperature, T_{max} , then rapidly decreased. The phase transition was accompanied by an increase in the partial specific volume of the hydrated polymer. This increase was significantly higher for solutions of PNIPAM in D₂O, compared to H₂O. A study by PPC of the phase transition of hydrophobically modified PNIPAM samples that undergo micellization in water demonstrated that the hydration of the polymeric micelles varies significantly as a function of the degree of hydrophobic substitution and length of the alkyl group linked to the polymer.

Lin L. N., Brandts J. F., Brandts J. M., and Plotnikov V. (2002) Determination of the volumetric properties of proteins and other solutes using pressure perturbation calorimetry. *Anal Biochem* **302**, 144-160.

Abstract: Pressure perturbation calorimetry is a new technique that measures the heat change in a solution that results when the pressure above the solution is changed. When used in a differential calorimeter containing a dilute solution of solute in the sample cell and the corresponding buffer in the reference cell, the measured differential heat can be used to calculate the thermal coefficient of expansion of the partial volume of the solute, α . For proteins in dilute aqueous solution, α is dominated by a temperature-dependent contribution arising from the interaction of protein groups with water at the protein-solvent interface. This arises due to the effect of the protein groups on the hydrogen-bonded structure of water, and thereby clearly differentiates between structure-making hydrophobic groups and structure-breaking hydrophilic groups. This solvation contribution to α can be accentuated in solvents having more structure (deuterium oxide) than water and attenuated in solvents having less structure (2.8 M guanidinium sulfate). Six different proteins (chymotrypsinogen, pepsinogen, lysozyme, bovine pancreatic trypsin inhibitor, ribonuclease A, and T4 lysozyme) were examined carefully by this technique, allowing estimates of various volumetric parameters including the volume change resulting from thermal unfolding of each protein. For ribonuclease A, results obtained in both water and deuterium oxide led to an estimate of the accessible surface area of the native protein of approximately 45% relative to the fully reduced unfolded protein. Also, it was also found that ligand binding to ribonuclease A led to changes in α , suggesting a burial of some surface area in the ligand-protein complex.

Mitra L., Smolin N., Ravindra R., Royer C., and Winter R. (2006) Pressure perturbation calorimetric studies of the solvation properties and the thermal unfolding of proteins in solution--experiments and theoretical interpretation. *Phys Chem Chem Phys* **8**, 1249-1265.

Abstract: We used pressure perturbation calorimetry (PPC), a relatively new and efficient technique, to study the solvation and volumetric properties of amino acids and peptides as well as of proteins in their native and unfolded state. In PPC, the coefficient of thermal expansion of the partial volume of the protein is deduced from the heat consumed or produced after small isothermal pressure jumps, which strongly depends on the interaction of the protein with the solvent or cosolvent at the protein-solvent interface. Furthermore, the effects of various chaotropic and kosmotropic cosolvents on the volume and expansivity changes of proteins were measured over a wide concentration range with high precision. Depending on the type of cosolvent and its concentration, specific differences were found for the solvation properties and unfolding behaviour of the proteins, and the volume change upon unfolding may even change sign. To yield a molecular interpretation of the different terms contributing to the partial protein volume and its temperature dependence, and hence a better understanding of the PPC data, molecular dynamics computer simulations on SNase were also carried out and compared with the experimental data. The PPC studies introduced aim to obtain more insight into the basic thermodynamic properties of protein solvation and volume effects accompanying structural transformations of proteins in various cosolvents on one hand, as these form the basis for understanding their physiological functions and their use in drug designing and formulations, but also to initiate further valuable applications in studies of other biomolecular and chemical systems.

Mitra L., Oleinikova A. and Winter R. (2008) Intrinsic Volumetric Properties of Alanine Isomers in Aqueous Solution. *Chemphyschem*.(epublication)

Abstract: The intrinsic volume and the intrinsic coefficient of thermal expansion of alanine tripeptides of different chirality are obtained from densimetric and pressure-perturbation calorimetric measurements by using the volumetric properties of water of hydration obtained from computer simulations. The aim of this study is to provide better understanding of the different contributions to the volumetric properties of peptides in solution. Water of hydration makes a major contribution to the volumetric properties measured experimentally. The intrinsic thermal expansivity of the peptides is found to be negative despite positive apparent values of thermal expansivity due to the large positive expansivity of the water of hydration, which notably exceeds the bulk value. The different volumetric behavior of the isomers is discussed in relation to their structural characteristics

Mitra L., Rouget J. B., Garcia-Moreno B., Royer C. A. and Winter R. (2008) Towards a Quantitative Understanding of Protein Hydration and Volumetric Properties. *Chemphyschem*.(epublication)

Abstract: Herein, we probe by pressure perturbation calorimetry (PPC) the coefficient of thermal expansion, the volumetric and the hydration properties of variants of a hyperstable variant of staphylococcal nuclease (SNase), Delta+PHS. The temperature-dependent volumetric properties of the folded and unfolded states of the wild-type protein are calculated with previously published data. The present PPC results are used to interpret the volume diagram and expansivity at a molecular level. We conclude that the expansivity of the unfolded state is, to a first approximation, temperature independent, while that of the folded state decreases with increasing temperature. Our data suggest that at low temperature the defining contribution to ΔV comes mainly from excluded volume differences and ΔV for unfolding is negative. In contrast, at high temperatures, differential solvation due to the increased exposed surface area of the unfolded state and, in particular, its larger thermal volume linked to the increased conformational dynamics of the unfolded state ensemble takes over and ΔV for unfolding eventually becomes positive

Nicolini C., Ravindra R., Ludolph B., and Winter R. (2004) Characterization of the temperature- and pressure-induced inverse and reentrant transition of the minimum elastin-like polypeptide GVG(VPGVG) by DSC, PPC, CD, and FT-IR spectroscopy. *Biophys J* **86**, 1385-1392.

Abstract: We investigated the temperature- and pressure-dependent structure and phase behavior of a solvated oligopeptide, GVG(VPGVG), which serves as a minimalistic elastin-like model system, over a large region of the thermodynamic phase field, ranging from 2 to 120 degrees C and from ambient pressure up to approximately 10 kbar, applying various spectroscopic (CD, FT-IR) and thermodynamic (DSC, PPC) measurements. We find that this octapeptide behaves as a two-state system which undergoes the well-

known inverse-temperature folding transition occurring at T approximately 36 degrees C, and, in addition, a slow trend reversal at higher temperatures, finally leading to a reentrant unfolding close to the boiling point of water. Furthermore, the pressure-dependence of the folding/unfolding transition was studied to yield a more complete picture of the p, T-stability diagram of the system. A molecular-level picture of these processes, in particular on the role of water for the folding and unfolding events of the peptide, presented with the help of molecular-dynamics simulations, is presented in a companion article in this issue.

Nicolini C., Kraineva J., Khurana M., Periasamy N., Funari S. S., and Winter R. (2006) Temperature and pressure effects on structural and conformational properties of POPC/SM/cholesterol model raft mixtures-- a FT-IR, SAXS, DSC, PPC and Laurdan fluorescence spectroscopy study. *Biochim Biophys Acta* **1758**, 248-258.

Abstract: We report on the effects of temperature and pressure on the structure, conformation and phase behavior of aqueous dispersions of the model lipid "raft" mixture palmitoyl-oleoylphosphatidylcholine (POPC)/bovine brain sphingomyelin (SM)/cholesterol (Chol) (1:1:1). We investigated interchain interactions, hydrogen bonding, conformational and structural properties as well as phase transformations of this system using Fourier transform-infrared (FT-IR) spectroscopy, small-angle X-ray scattering (SAXS), differential scanning calorimetry (DSC) coupled with pressure perturbation calorimetry (PPC), and Laurdan fluorescence spectroscopy. The IR spectral parameters in combination with the scattering patterns from the SAXS measurements were used to detect structural and conformational transformations upon changes of pressure up to 7-9 kbar and temperature in the range from 1 to about 80 degrees C. The generalized polarization function (GP) values, obtained from the Laurdan fluorescence spectroscopy studies also reveal temperature and pressure dependent phase changes. DSC and PPC were used to detect thermodynamic properties accompanying the temperature-dependent phase changes. In combination with literature fluorescence spectroscopy and microscopy data, a tentative p,T stability diagram of the mixture has been established. The data reveal a broad liquid-order/solid-ordered (lo+so) two-phase coexistence region below 8+/-2 degrees C at ambient pressure. With increasing temperature, a lo+ld+so three-phase region is formed, which extends up to approximately 27 degrees C, where a liquid-ordered/liquid-disordered (lo+ld) immiscibility region is formed. Finally, above 48+/-2 degrees C, the POPC/SM/Chol (1:1:1) mixture becomes completely fluid-like (liquid-disordered, ld). With increasing pressure, all phase transition lines shift to higher temperatures. Notably, the lo+ld (+so) phase coexistence region, mimicking raft-like lateral phase separation in natural membranes, extends over a rather wide temperature range of about 40 degrees C, and a pressure range, which extends up to about 2 kbar for T=37 degrees C. Interestingly, in this pressure range, ceasing of membrane protein function in natural membrane environments has been observed for a variety of systems.

Nicolini C., Celli A., Gratton E., and Winter R. (2006) Pressure tuning of the morphology of heterogeneous lipid vesicles: a two-photon-excitation fluorescence microscopy study. *Biophys J* **91**, 2936-2942.

Abstract: We used a technique that allows us to visualize local and morphological changes of the membrane of more component giant unilamellar vesicles due to high pressure perturbation. Under these conditions, thermally induced processes are largely suppressed, and the bending rigidity and line tension are influenced by pressure-induced changes in lipid molecular packing and shape only. We studied the effect of pressure on the lateral organization and morphology of the model raft system DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine)/sphingomyelin/cholesterol as well as of the fluid mixture POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine)/DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine) by two-photon excitation fluorescence microscopy. The pressure-dependent experiments were carried out using a sample cell made from a thin fused silica capillary. The use of Laurdan as fluorescence label allowed us to also follow the lipid phase state by calculating the generalized polarization (GP) values of the vesicles and extracting their average value. During the compression cycle, a reduction in the volume of the vesicles is observed, accompanied by an increase of the average GP value, indicating an increasingly tighter packing of the lipids. Interestingly, the two systems studied show phenomena of budding and fission, and these at surprisingly low pressures of 200-300 bar. Moreover, these budding processes are not directly related to phase transitions to an overall ordered conformational state of the lipid membrane, which occur at much higher pressures. The topological changes of the lipid vesicles are irreversible and exhibit a different behavior depending on whether the pressure is increased or decreased. The results are discussed in light of the various contributions to the free energy functional of lipid vesicles. Finally, the biological relevance of these studies is highlighted.

Ravindra R., Zhao S., Gies H., and Winter R. (2004) Protein encapsulation in mesoporous silicate: the effects of confinement on protein stability, hydration, and volumetric properties. *J Am Chem Soc* **126**, 12224-12225.

Ravindra R. and Winter R. (2004) Pressure perturbation calorimetry: a new technique provides surprising results on the effects of co-solvents on protein solvation and unfolding behaviour. *Chemphyschem* **5**, 566-571.

Ravindra, R., and Winter, R. (2003) On the temperature=pressure free energy landscape of proteins. *Chemphyschem* **4**, 359-365.

Abstract: We studied the thermodynamic stability of a small monomeric protein, staphylococcal nuclease (Snase), as a function of both temperature and pressure, and expressed it as a 3D free-energy surface on the p,T-plane using a second-order Taylor expansion of the Gibbs free-energy change ΔG upon unfolding. We took advantage of a series of different techniques (small-angle X-ray scattering, Fourier-transform infrared spectroscopy, differential thermal analysis, pressure perturbation calorimetry and densitometry) in the evaluation of the conformation of the protein and in evaluating the changes in the thermodynamic parameters upon unfolding, such as the heat capacity, enthalpy, entropy, volume, isothermal compressibility and expansivity. The calculated results of the free-energy landscape of the protein are in good agreement with experimental data of the p,T-stability diagram of the protein over a temperature range from 200 to 400 K and at pressures from ambient pressure to 4000 bar. The results demonstrate that combined temperature \pm pressure-dependent studies can help delineate the free-energy landscape of proteins and hence help elucidate which features and thermodynamic parameters are essential in determining the stability of the native conformational state of proteins. The approach presented may also be used for studying other systems with so-called re-entrant or Tamman loopshaped phase diagrams.

Royer C.A. (2005) Insights into the role of hydration in protein structure and stability obtained through hydrostatic pressure studies. *Braz J Med Biol Res.* **38**, 1167-73.

Abstract: A thorough understanding of protein structure and stability requires that we elucidate the molecular basis for the effects of both temperature and pressure on protein conformational transitions. While temperature effects are relatively well understood and the change in heat capacity upon unfolding has been reasonably well parameterized, the state of understanding of pressure effects is much less advanced. Ultimately, a quantitative parameterization of the volume changes (at the basis of pressure effects) accompanying protein conformational transitions will be required. The present report introduces a qualitative hypothesis based on available model compound data for the molecular basis of volume change upon protein unfolding and its dependence on temperature.

Smolin N. and Winter R. (2006) A molecular dynamics simulation of SNase and its hydration shell at high temperature and high pressure. *Biochim Biophys Acta* **1764**, 522-534.

Abstract: Temperature- and pressure-induced unfolding of staphylococcal nuclease (SNase) was studied by Royer, Winter et al. using a variety of experimental techniques (SAXS, FT-IR and fluorescence spectroscopy, DSC, PPC, densimetry). For a more detailed understanding of the underlying mechanistic processes of the different unfolding scenarios, we have carried out a series of molecular dynamics (MD) computer simulations on SNase. We investigated the initial changes of the structure of the protein upon application of pressure (up to 5 kbar) and discuss volumetric and structural differences between the native and pressure pre-denatured state. Additionally, we have obtained the compressibility of the protein and hydration water and compare these data with experimental results. As water plays a crucial role in determining the structure, dynamics and function of proteins, we undertook a detailed analysis of the structure of the interfacial water and the protein-solvent H-bond network as well. Moreover, we report here also MD results on the temperature-induced unfolding of SNase. The time evolution of the protein volume and solvent accessible surface area during thermal unfolding have been investigated, and we present a detailed discussion of the temperature-induced unfolding pathway of SNase in terms of secondary and tertiary structural changes.

Seeger H. M., Gudmundsson M. L. and Heimburg T. (2007) How anesthetics, neurotransmitters, and antibiotics influence the relaxation processes in lipid membranes. *J Phys. Chem B* **111**, 13858-13866.

Abstract: We used pressure perturbation calorimetry to investigate the relaxation time scale after a jump into the melting transition regime of artificial lipid membranes. This time is equivalent to the characteristic

rate of domain growth. The studies were performed on single-component large unilamellar and multilamellar vesicle systems with and without the addition of small molecules such as general anesthetics, neurotransmitters, and antibiotics. These drugs interact with membranes and affect melting points and profiles. In all systems, we found that heat capacity and relaxation times are linearly related to each other in a simple manner, and we outline the theoretical origin of this finding. Thus, the influence of a drug on the time scale of domain formation processes can be understood on the basis of their influence on the heat capacity profile. This allows estimations of the characteristic relaxation time scales in biological membranes.

Senear D. F., Tretyachenko-Ladokhina V., Opel M. L., Aeling K. A., Hatfield G. W., Franklin L. M., Darlington R. C. and Alexander Ross J. B. (2007) Pressure dissociation of integration host factor-DNA complexes reveals flexibility-dependent structural variation at the protein-DNA interface. *Nucleic Acids Res* **35**, 1761-1772.

Abstract: E. coli Integration host factor (IHF) condenses the bacterial nucleoid by wrapping DNA. Previously, we showed that DNA flexibility compensates for structural characteristics of the four consensus recognition elements associated with specific binding (Aeling et al., J. Biol. Chem. 281, 39236-39248, 2006). If elements are missing, high-affinity binding occurs only if DNA deformation energy is low. In contrast, if all elements are present, net binding energy is unaffected by deformation energy. We tested two hypotheses for this observation: in complexes containing all elements, (1) stiff DNA sequences are less bent upon binding IHF than flexible ones; or (2) DNA sequences with differing flexibility have interactions with IHF that compensate for unfavorable deformation energy. Time-resolved Forster resonance energy transfer (FRET) shows that global topologies are indistinguishable for three complexes with oligonucleotides of different flexibility. However, pressure perturbation shows that the volume change upon binding is smaller with increasing flexibility. We interpret these results in the context of Record and coworker's model for IHF binding (J. Mol. Biol. 310, 379-401, 2001). We propose that the volume changes reflect differences in hydration that arise from structural variation at IHF-DNA interfaces while the resulting energetic compensation maintains the same net binding energy.

Smolin N. and Winter R. (2006) A molecular dynamics simulation of SNase and its hydration shell at high temperature and high pressure. *Biochim Biophys Acta* **1764**, 522-534.

Abstract: Temperature- and pressure-induced unfolding of staphylococcal nuclease (SNase) was studied by Royer, Winter et al. using a variety of experimental techniques (SAXS, FT-IR and fluorescence spectroscopy, DSC, PPC, densimetry). For a more detailed understanding of the underlying mechanistic processes of the different unfolding scenarios, we have carried out a series of molecular dynamics (MD) computer simulations on SNase. We investigated the initial changes of the structure of the protein upon application of pressure (up to 5 kbar) and discuss volumetric and structural differences between the native and pressure pre-denatured state. Additionally, we have obtained the compressibility of the protein and hydration water and compare these data with experimental results. As water plays a crucial role in determining the structure, dynamics and function of proteins, we undertook a detailed analysis of the structure of the interfacial water and the protein-solvent H-bond network as well. Moreover, we report here also MD results on the temperature-induced unfolding of SNase. The time evolution of the protein volume and solvent accessible surface area during thermal unfolding have been investigated, and we present a detailed discussion of the temperature-induced unfolding pathway of SNase in terms of secondary and tertiary structural changes.

Wang S. L. and Epanand R. M. (2004) Factors determining pressure perturbation calorimetry measurements: evidence for the formation of metastable states at lipid phase transitions. *Chem Phys Lipids* **129**, 21-30.

Abstract: The factors that influence the application of pressure perturbation calorimetry in studying the volume change of the phase transition of lipids are discussed. These factors include a correction for the temperature-shift induced by perturbation, the kinetic irreversibility of the phase transition and the magnitude of the pressure perturbation. We take into account the fact that the dependence of the phase transition temperature on pressure will affect the temperature-shift induced by pressure. As a result, there is a discrepancy between the compression part of the cycle and the expansion. In addition, sequential cycles lead to a gradual loss in magnitude of the heat effect upon pressure perturbation. We suggest that these

phenomena can be explained by the formation of a metastable glass-like state that converts to a stable phase at temperatures removed from the region of the phase transition.

Zorrilla S., Chaix D., Ortega A., Alfonso C., Doan T., Margeat E., Rivas G., Aymerich S., Declerck N. and Royer C. A. (2007) Fructose-1,6-bisphosphate Acts Both as an Inducer and as a Structural Cofactor of the Central Glycolytic Genes Repressor (CggR). *Biochemistry* **46**, 14996-15008.

Abstract: CggR is the transcriptional repressor of the gapA operon encoding central glycolytic enzymes in *Bacillus subtilis*. Recently, a detailed mechanistic characterization of gapA induction revealed that the binding of fructose-1,6-bisphosphate (FBP) to a low affinity site on CggR ($K_d > 100 \mu\text{M}$) is responsible for repressor release from the DNA. In addition, this prior work demonstrated that FBP binds to a second high affinity site on the repressor, causing a conformational change in the CggR/DNA complexes, but with no consequence on CggR affinity for its operator DNA. In the present study we have thoroughly analyzed the structural and thermodynamic consequences of FBP binding to CggR. Results of fluorescence anisotropy titrations, calorimetry and limited proteolysis confirm the existence in CggR of a high affinity site for FBP, with a K_d of around 6 μM . Using analytical size-exclusion chromatography, ultracentrifugation as well as fluorescence correlation spectroscopy (FCS) and pressure perturbation, we show that FBP binding at this site reduces the size of the CggR oligomers and induces conformational changes that stabilize the dimer against denaturation. Hence, FBP has a dual role on CggR structure and regulatory function. In addition to acting as an inducer of transcription at the low affinity site, FBP bound to the high affinity site acts as a structural cofactor for the repressor, with profound effects on its quaternary structure as well as on its conformational dynamics and stability. This high affinity FBP site apparently evolved from the sugar substrate binding site of homologous enzymes.