

DSC VIII: Protein-Small Molecule Interactions (drugs, ligands, etc)

Amornwittawat N., Wang S., Banatiao J., Chung M., Velasco E., Duman J. G. and Wen X. (2008) Effects of polyhydroxy compounds on beetle antifreeze protein activity. *Biochim Biophys Acta*.(epublication)

Abstract: Antifreeze proteins (AFPs) noncolligatively depress the nonequilibrium freezing point of a solution and produce a difference between the melting and freezing points termed thermal hysteresis (TH). Some low-molecular-mass solutes can affect the TH values. The TH enhancement effects of selected polyhydroxy compounds including polyols and carbohydrates on an AFP from the beetle *Dendroides canadensis* were systematically investigated using differential scanning calorimetry (DSC). The number of hydroxyl groups dominates the molar enhancement effectiveness of polyhydroxy compounds having one to five hydroxyl groups. However, the above rule does not apply for polyhydroxy compounds having more than five hydroxyl groups. The most efficient polyhydroxy enhancer identified is trehalose. In a combination of enhancers the strongest enhancer plays the major role in determining the TH enhancement. Mechanistic insights into identification of highly efficient AFP enhancers are provided

Amornwittawat N., Wang S., Duman J. G. and Wen X. (2008) Polycarboxylates enhance beetle antifreeze protein activity. *Biochim Biophys Acta* **1784**, 1942-1948.

Abstract: Antifreeze proteins (AFPs) lower the noncolligative freezing point of water in the presence of ice below the ice melting point. The temperature difference between the melting point and the noncolligative freezing point is termed thermal hysteresis (TH). The magnitude of the TH depends on the specific activity and the concentration of AFP, and the concentration of enhancers in the solution. Known enhancers are certain low molecular mass molecules and proteins. Here, we investigated a series of polycarboxylates that enhance the TH activity of an AFP from the beetle *Dendroides canadensis* (DAFP) using differential scanning calorimetry (DSC). Triethylenetetramine-N,N,N',N'',N''',N''''-hexaacetate, the most efficient enhancer identified in this work, can increase the TH of DAFP by nearly 1.5 fold over than that of the published best enhancer, citrate. The Zn(2+) coordinated carboxylate results in loss of the enhancement ability of the carboxylate on antifreeze activity. There is not an additional increase in TH when a weaker enhancer is added to a stronger enhancer solution. These observations suggest that the more carboxylate groups per enhancer molecule the better the efficiency of the enhancer and that the freedom of motion of these molecules is necessary for them to serve as enhancers for AFP. The hydroxyl groups in the enhancer molecules can also positively affect their TH enhancement efficiency, though not as strongly as carboxylate groups. Mechanisms are discussed

Andersen K. K., Westh P. and Otzen D. E. (2008) Global study of myoglobin-surfactant interactions. *Langmuir* **24**, 399-407.

Abstract: Surfactants interact with proteins in multifarious ways which depend on surfactant concentration and structure. To obtain a global overview of this process, we have analyzed the interaction of horse myoglobin (Mb) with an anionic (SDS) and cationic (CTAC) surfactant, using both equilibrium titration techniques and stopped-flow kinetics. Binding and kinetics of conformational changes can be divided into a number of different regions (five below the cmc and one above) with very distinct features (broadly similar between the two surfactants, despite their difference in head group and chain length), which nuance the classical view of biphasic binding prior to micellization. In stage A, fairly weak interactions lead to a linear decrease in thermal stability. This gives way to a more cooperative process in stage B, where aggregates (presumably hemimicelles) start to form on the protein surface, leading to global denaturation (loss of a thermal transition) and biphasic unfolding kinetics. This is consolidated in stage C with titratable surfactant adsorption. Adsorption of this surfactant species leads to significant changes in kinetics, namely, inhibition of unfolding kinetics in CTAC and altered unfolding amplitudes in SDS, though the process is still biphasic in both surfactants. Stage D commences the reduction in exothermic binding signals, leading to further uptake of 5 (SDS) or 31 (CTAC) surfactant molecules without any major changes in protein conformation. In stage E many more surfactant molecules (46 SDS and 39 CTAC) are bound, presumably as quasi-micellar structures, and we observe a very slow unfolding phase in SDS, which disappears as we reach the cmc. Above the cmc, the unfolding rates remain essentially constant in SDS, but increase significantly in CTAC, possibly because binding of bulk micelles removes the inhibition by hemimicellar aggregates. Our work highlights the fascinating richness of conformational changes that proteins can undergo in the presence of molecules with self-assembling properties

Anteono C., Rodahl A. M., Meiering E., Heynen M. L., Sennisterra G. A., and Lepock J. R. (1994) Interaction of dibucaine with the transmembrane domain of the Ca(2+)-ATPase of sarcoplasmic reticulum. *Biochemistry* **33**, 12283-12290.

Abstract: The site of interaction of dibucaine with the Ca(2+)-ATPase of rabbit sarcoplasmic reticulum, an ion-transporting membrane protein, was investigated by determining the effect of dibucaine on the denaturation of the transmembrane domain and the aqueous domain containing, respectively, the high-affinity Ca²⁺ binding sites and the site of ATP hydrolysis. In the absence of Ca²⁺, a single irreversible denaturation transition with T_m approximately equal to 49 degrees C is observed for the Ca(2+)-ATPase by differential scanning calorimetry (DSC). In the presence of Ca²⁺, but not Mg²⁺, Sr²⁺, or Ba²⁺, a new high-temperature transition is observed that has been shown to be due to stabilization of the transmembrane region [Lepock, J. R., Rodahl, A. M., Zhang, C., Heynen, M. L., Waters, B., & Cheng, K. H. (1990) *Biochemistry* **29**, 681-689]. The maximum stabilization corresponds to a shift in T_m of 13.8 degrees C, and Hill analysis indicates that the Ca²⁺ binding site yielding stabilization has a K_d = 2.5 x 10⁻⁴ M with a cooperativity (n) of 1. Thus, stabilization is due to Ca²⁺ binding not to the high-affinity sites but to one of the previously observed sites of low or intermediate affinity, which must be located in the transmembrane or stalk subdomains. Dibucaine has little effect on the T_m of the aqueous domain, but it decreases the T_m of the transmembrane domain with K_d approximately equal to 4.1 x 10⁻⁴ M and a cooperativity of approximately 1.6, implying that destabilization is due to the binding of dibucaine to sites of intermediate or moderately high affinity.(ABSTRACT TRUNCATED AT 250 WORDS).

Antharam V. C., Farver R. S., Kuznetsova A., Sippel K. H., Mills F. D., Elliott D. W., Sternin E. and Long J. R. (2008) Interactions of the C-terminus of lung surfactant protein B with lipid bilayers are modulated by acyl chain saturation. *Biochim Biophys Acta* **1778**, 2544-2554.

Abstract: Lung surfactant protein B (SP-B) is critical to minimizing surface tension in the alveoli. The C-terminus of SP-B, residues 59-80, has much of the surface activity of the full protein and serves as a template for the development of synthetic surfactant replacements. The molecular mechanisms responsible for its ability to restore lung compliance were investigated with circular dichroism, differential scanning calorimetry, and (31)P and (2)H solid-state NMR spectroscopy. SP-B(59-80) forms an amphipathic helix which alters lipid organization and acyl chain dynamics in fluid lamellar phase 4:1 DPPC:POPG and 3:1 POPC:POPG MLVs. At higher levels of SP-B(59-80) in the POPC:POPG lipid system a transition to a nonlamellar phase is observed while DPPC:POPG mixtures remain in a lamellar phase. Deuterium NMR shows an increase in acyl chain order in DPPC:POPG MLVs on addition of SP-B(59-80); in POPC:POPG MLVs, acyl chain order parameters decrease. Our results indicate SP-B(59-80) penetrates deeply into DPPC:POPG bilayers and binds more peripherally to POPC:POPG bilayers. Similar behavior has been observed for KL(4), a peptide mimetic of SP-B which was originally designed using SP-B(59-80) as a template and has been clinically demonstrated to be successful in treating respiratory distress syndrome. The ability of these helical peptides to differentially partition into lipid lamellae based on their degree of monounsaturations and subsequent changes in lipid dynamics suggest a mechanism for lipid organization and trafficking within the dynamic lung environment

Antipova A. S., Semenova M. G., Belyakova L. E., and Il'in M. M. (2001) On relationships between molecular structure, interaction and surface behavior in mixture: small-molecule surfactant+protein. *Colloids Surf B Biointerfaces* **21**, 217-230.

Abstract: We report on the effect of distinct in nature small-molecule surfactants (model, a sodium salt of capric acid, Na-caprate; and commercially important, a citric acid ester of monoglyceride, CITREM; a sodium salt of stearyl-lactoyl lactic acid, SSL (Na(+)); polyglycerol ester, PGE (080)) on molecular properties in a bulk and at the air-water interface of globular legumin and random-coiled micellar sodium caseinate. The role of the structure of both proteins and small-molecule surfactants in the effect studied has been elucidated by measurements in a bulk aqueous medium of the enthalpy of their interaction from mixing calorimetry, the change in value of weight average molecular weight of the proteins and the thermodynamics of the pair protein-protein interactions from laser static light scattering as well as, in addition, by measurements of the change in hydrodynamic radius for micellar sodium caseinate from laser dynamic light scattering. The effect of the small-molecule surfactants on the thermodynamics of the protein heat denaturation and thereby on the protein conformational stability has been studied by differential scanning calorimetry in the case of globular legumin. The interrelation between the effects of the small-molecule surfactants on the properties of the proteins in a bulk and at the planar air-water interface has

been elucidated by tensiometry. The combined data of mixing calorimetry, differential scanning calorimetry and laser light scattering suggest some complex formation between the small-molecule surfactants and the proteins in a bulk aqueous medium. Predominantly hydrophobic interaction along with electrostatic and hydrogen bonding form the basis of the complex formation. The found effect of the small-molecule surfactants on the surface activity of their mixtures with proteins is governed primarily by both the extent of the protein association, resulting in specific hydrophobicity/hydrophilicity of the surface of the protein associates, and the specific protein conformational stability, for the globular protein, produced by the interaction between the proteins and the small-molecule surfactants.

Barcelo F., Ortiz-Lombardia M., and Portugal J. (2001) Heterogeneous DNA binding modes of berenil. *Biochim Biophys Acta* **1519**, 175-184.

Abstract: Isothermal titration calorimetry (ITC) profiles of berenil bound to different DNAs show that, despite the strong preference of berenil for AT-rich regions in DNA, it can bind to other DNA sequences significantly. The ITC results were used to quantify the binding of berenil, and the thermodynamic profiles were obtained using natural DNAs as well as synthetic polynucleotides. ITC binding isotherms cannot be simply described when a single set of identical binding sites is considered, except for poly[d(A-T)₂]. Ultraviolet melting of DNA and differential scanning calorimetry were also used to quantify several aspects of the binding of berenil to salmon testes DNA. We present evidence for secondary binding sites for berenil in DNA, corresponding to G+C rich sites. Berenil binding to poly[d(G-C)₂] is also observed. Circular dichroism experiments showed that binding to GC-rich sites involves drug intercalation. Using a molecular modeling approach we demonstrate that intercalation of berenil into CpG steps is sterically feasible.

Bekker E. G., Creagh A. L., Sanaie N., Yumoto F., Lau G. H., Tanokura M., Haynes C. A., and Murphy M. E. (2004) Specificity of the synergistic anion for iron binding by ferric binding protein from *Neisseria gonorrhoeae*. *Biochemistry* **43**, 9195-9203.

Abstract: Ferric binding protein in *Neisseria gonorrhoeae* (nFbpA) transports iron from outer membrane receptors for host proteins across the periplasm to a permease in an alternative pathway to the use of siderophores in some pathogenic bacteria. Phosphate and nitrilotriacetate, both at pH 8, and vanadate at pH 9 are shown to be synergistic in promoting ferric binding to nFbpA, in contrast to carbonate and sulfate. Interestingly, only phosphate produces the fully closed conformation of nFbpA as defined by native electrophoresis. The role of phosphate was probed by constructing three mutants: Q58E, Q58R, and G140H. The anion and iron binding properties of the Q58E mutant are similar to the wild-type protein, implying that one phosphate oxygen is a hydrogen bond donor and may in part define the specificity of nFbpA for phosphate over sulfate. Phosphate is a weakly synergistic anion in the Q58R and G140H mutants, and these mutants do not form completely closed structures. Ferric binding was investigated by both isothermal titration and differential scanning calorimetry. The apparent affinity of nFbpA for iron in a solution of 30 mM citrate is 1 order of magnitude larger in the presence ($K_{app} = 1.7 \times 10^5 M^{-1}$) of phosphate than in its absence ($K_{app} = 1.6 \times 10^4 M^{-1}$) at pH 7. Similar results were obtained at pH 8. This increase in affinity with phosphate as well as the formation of closed structure allows nFbpA to compete for free ferric ions in solution and suggests that ferric binding to nFbpA is regulated by the synergistic phosphate anion at sites of iron uptake.

Błaszczuk U. and Wasylewski Z. (2003) Interaction of cAMP receptor protein from *Escherichia coli* with cAMP and DNA studied by differential scanning calorimetry. *J Protein Chem* **22**, 285-293.

Abstract: The cyclic AMP receptor protein (CRP) regulates the expression of many genes in *Escherichia coli*. The protein is a homodimer, and each monomer is folded into two distinct structural domains. In this study, we have used differential scanning calorimetry (DSC) and circular dichroism (CD) to measure the enthalpy change and melting temperature of the apo-CRP and CRP complexes with cAMP or DNA sequences lac, gal, and palindromic ICAP. DSC and CD measurements showed irreversible thermal denaturation process of CRP. Enthalpy of dissociation of the protein-DNA complex, as measured by DSC, depends on the DNA sequence. The thermal transition of the protein in CRP-DNA complexes, measured by CD, indicates that the protein stability in the complex is also DNA sequence-dependent.

Boeckler F. M., Joerger A. C., Jaggi G., Rutherford T. J., Veprintsev D. B. and Fersht A. R. (2008) Targeted rescue of a destabilized mutant of p53 by an in silico screened drug. *Proc. Natl. Acad. Sci U. S. A* **105**, 10360-10365.

Abstract: The tumor suppressor p53 is mutationally inactivated in approximately 50% of human cancers. Approximately one-third of the mutations lower the melting temperature of the protein, leading to its rapid denaturation. Small molecules that bind to those mutants and stabilize them could be effective anticancer drugs. The mutation Y220C, which occurs in approximately 75,000 new cancer cases per annum, creates a surface cavity that destabilizes the protein by 4 kcal/mol, at a site that is not functional. We have designed a series of binding molecules from an in silico analysis of the crystal structure using virtual screening and rational drug design. One of them, a carbazole derivative (PhiKan083), binds to the cavity with a dissociation constant of approximately 150 μ M. It raises the melting temperature of the mutant and slows down its rate of denaturation. We have solved the crystal structure of the protein-PhiKan083 complex at 1.5-Å resolution. The structure implicates key interactions between the protein and ligand and conformational changes that occur on binding, which will provide a basis for lead optimization. The Y220C mutant is an excellent "druggable" target for developing and testing novel anticancer drugs based on protein stabilization. We point out some general principles in relationships between binding constants, raising of melting temperatures, and increase of protein half-lives by stabilizing ligands

Booth V. K., Roberts J. C., Warters R. L., Wilmore B. H., and Lepock J. R. (2000) Radioprotective thiolamines WR-1065 and WR-33278 selectively denature nonhistone nuclear proteins. *Radiat Res* **153**, 813-822.

Abstract: Differential scanning calorimetry was used to study the interactions of nuclei isolated from Chinese hamster V79 cells with the radioprotector WR-1065, other thiol compounds, and polyamines. Differential scanning calorimetry monitors denaturation of macromolecules and resolves the major nuclear components (e.g. constrained and relaxed DNA, nucleosome core, and nuclear matrix) of intact nuclei on the basis of thermal stability. WR-1065 treatment (0.5-10 mM) of isolated nuclei led to the irreversible denaturation of nuclear proteins, a fraction of which are nuclear matrix proteins. Denaturation of 50% of the total nonhistone nuclear protein content of isolated nuclei occurred after exposure to 4.7 mM WR-1065 for 20 min at 23 degrees C. In addition, a 22% increase in the insoluble protein content of nuclei isolated from V79 cells that had been treated with 4 mM WR-1065 for 30 min at 37 degrees C was observed, indicating that WR-1065-induced protein denaturation occurs not only in isolated nuclei but also in the nuclei of intact cells. From the extent of the increase in insoluble protein in the nucleus, protein denaturation by WR-1065 is expected to contribute to drug toxicity at concentrations greater than approximately 4 mM. WR-33278, the disulfide form of WR-1065, was approximately twice as effective as the free thiol at denaturing nuclear proteins. The proposed mechanism for nucleoprotein denaturation is through direct interactions with protein cysteine groups with the formation of destabilizing protein-WR-1065 disulfides. In comparison to its effect on nuclear proteins in isolated nuclei, WR-1065 had only a very small effect on non-nuclear proteins of whole cells, isolated nuclear matrix, or the thiol-rich Ca(2+)ATPase of sarcoplasmic reticulum, indicating that WR-1065 can effectively denature protein only inside an intact nucleus, probably due to the increased concentration of the positively charged drug in the vicinity of DNA.

Brandts J. F. and Lin L. N. (1990) Study of strong to ultratight protein interactions using differential scanning calorimetry. *Biochemistry* **29**, 6927-6940.

Abstract: Data from differential scanning calorimetry (DSC) may be used to estimate very large binding constants that cannot be conveniently measured by more conventional equilibrium techniques. Thermodynamic models have been formulated to describe interacting systems that involve either one thermal transition (protein-ligand) or two thermal transitions (protein-protein) and either 1:1 or higher binding stoichiometry. Methods are described for obtaining binding constants and heats of binding by two different methods: calculation or simulation fitting of data. Extensive DSC data on 2'CMP binding to RNase are presented and analyzed by the two methods. It is found that the methods agree when binding sites are completely saturated, but substantial errors arise in the calculation method when site saturation is incomplete and the transition of liganded molecules overlaps that of unliganded molecules. This arises primarily from an inability to determine T_M (i.e., the temperature where concentrations of folded and unfolded protein are equal) under weak-binding conditions. Results from simulation show that the binding constants and heats of binding from the DSC method agree quantitatively with corresponding estimates obtained from equilibrium methods when extrapolated to the same temperature. It was also found from the DSC data that the binding constant decreases with increasing concentration of ligand, which might arise from nonideality effects associated with dimerization of 2'CMP. Simulations show that the DSC method is capable of estimating binding constants for ultratight interactions up to perhaps 10^{40} M^{-1} or higher, while

most equilibrium methods fail well below 10^{10} M^{-1} . DSC data from the literature on a number of interacting systems (trypsin-soybean trypsin inhibitor, trypsin-ovomucoid, trypsin-pancreatic trypsin inhibitor, chymotrypsin-subtilisin inhibitor, subtilisin BPN-subtilisin inhibitor, RNase S protein-RNase S peptide, avidin-biotin, ovotransferrin- Fe^{3+} , superoxide dismutase- Zn^{2+} , alkaline phosphatase- Zn^{2+} , and assembly of regulatory and catalytic subunits of aspartate transcarbamoylase) were analyzed by simulation fitting or by calculation. Apparent single-site binding constants ranged from ca. 10^5 to 10^{20} M^{-1} , while the interaction constant for assembly of aspartate transcarbamoylase was estimated as 10^{37} in molarity units. For most of these systems, the DSC interaction constants compared favorably with other literature estimates, for some it did not for reasons unknown, while for still others this represented the first estimate. Simulations show that for proteins having two binding sites for the same ligand within a single cooperative unit, ligand rearrangement will occur spontaneously during a DSC scan as the transition temperature of the unliganded protein is approached.(ABSTRACT TRUNCATED AT 400 WORDS).

Brass O., Letoffe J. M., Bakkali A., Bureau J. C., Corot C., and Claudy P. (1995) Involvement of protein solvation in the interaction between a contrast medium (iopamidol) and fibrinogen or lysozyme. *Biophys Chem* **54**, 83-94.

Abstract: The interaction between proteins and a radiological commonly-used contrast medium (iopamidol) have been studied by calorimetry. When aqueous solutions of fibrinogen or of lysozyme (20 g/l) are mixed with an aqueous solution of iopamidol (1,3-5 benzendicardoxamid,N,N'-bis[2-hydroxy-1-(hydroxymethyl)ethyl]-5- [(2-hydroxy-1-oxopropyl)amino]-2,4,6-triiodo) in the clinical blood concentration range (26-485 mM), isothermal calorimetry reveals a weak endothermic interaction at a high concentration of iopamidol for both proteins. This endothermic effect does not appear to be due to direct protein-iopamidol association. Differential scanning calorimetry confirms the influence of iopamidol by the change in protein unfolding in the presence of contrast medium, and suggests alterations in the protein solvation as a mechanism. Dilution studies indicate that iopamidol can influence protein solvation even when water molecules are present in a molecular excess of 1000. The influence of iopamidol on the availability of water molecules and the absence of direct interaction with the protein molecules is shown by Raman spectroscopy of two amino acids in the presence of iopamidol. The spectrum of alanine is unchanged at any iopamidol concentration studied, whereas the spectrum lines due to the thiol group of cysteine are shifted in a manner consistent with altered solvation.

Celej M. S., Dassie S. A., Freire E., Bianconi M. L., and Fidelio G. D. (2005) Ligand-induced thermostability in proteins: Thermodynamic analysis of ANS-albumin interaction. *Biochim Biophys Acta* **1750**, 122-133.

Abstract: A comparative thermodynamic study of the interaction of anilinonaphthalene sulfonate (ANS) derivatives with bovine serum albumin (BSA) was performed by using differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC). The chemically related ligands, 1,8-ANS and 2,6-ANS, present a similar affinity for BSA with different binding energetics. The analysis of the binding driving forces suggests that not only hydrophobic effect but also electrostatic interactions are relevant, even though they have been extensively used as probes for non-polar domains in proteins. Ligand association leads to an increase in protein thermostability, indicating that both dyes interact mainly with native BSA. ITC data show that 1,8-ANS and 2,6-ANS have a moderate affinity for BSA, with an association constant of around $1-9 \times 10^5 \text{ M}^{-1}$ for the high-affinity site. Ligand binding is disfavoured by conformational entropy. The theoretical model used to simulate DSC data satisfactorily reproduces experimental thermograms, validating this approach as one which provides new insights into the interaction between one or more ligands with a protein. By comparison with 1,8-ANS, 2,6-ANS appears as a more "inert" probe to assess processes which involve conformational changes in proteins.

Celej M. S., Dassie S. A., Gonzalez M., Bianconi M. L., and Fidelio G. D. (2006) Differential scanning calorimetry as a tool to estimate binding parameters in multiligand binding proteins. *Anal Biochem* **350**, 277-284.

Abstract: The stability of proteins and their interactions with other molecules is a topic of special interest in biochemistry because many cellular processes depend on that. New methods and approaches are constantly developed to elucidate the energetics of biomolecular recognition. In this sense, the application of the theory of macromolecular unfolding linked to ligand binding to differential scanning calorimetry (DSC) has proved to be a useful tool to simultaneously characterize the energetics of unfolding and

binding. Although the general theory is well known, the applicability of DSC to study the interaction of biomolecules is not common. In the current work, we estimated the binding parameters of 8-anilinoanthracene-1-sulfonic acid to human serum albumin using DSC. This model system was chosen due to both the complex stoichiometry and the moderate binding constants. From DSC curves acquired at different ligand concentrations, we obtained the number of bound ligands, the binding constants, and the binding enthalpy for each independent binding site. Compared with those parameters determined by titration calorimetry, the results highlight the potentiality of DSC to estimate binding parameters in multiligand binding proteins.

Christensen T., Svensson B., and Sigurskjold B. W. (1999) Thermodynamics of reversible and irreversible unfolding and domain interactions of glucoamylase from *Aspergillus niger* studied by differential scanning and isothermal titration calorimetry. *Biochemistry* **38**, 6300-6310.

Abstract: The stability of three forms of glucoamylase from *Aspergillus niger* has been investigated by differential scanning and isothermal titration calorimetry: Glucoamylase 1 (GA1), which consists of a catalytic domain and a starch-binding domain (SBD) connected by a heavily O-glycosylated linker region; glucoamylase 2 (GA2), which lacks SBD; and a proteolytically cleaved glucoamylase (GACD), which contains the catalytic domain and part of the linker region. The structures of the catalytic domain with part of the linker region and of SBD are known from crystallography and NMR, respectively, but the precise spatial arrangement of the two domains in GA1 is unknown. To investigate the stability of the three glucoamylase forms, we unfolded the enzymes thermally by differential scanning calorimetry (DSC). Aggregation occurs upon heating GA1 and GA2 at pH values between 2.5 and 5.0, whereas no aggregation is observed at higher pH (5.5-7.5). At all pH values, the catalytic domain of GA1 and GA2 unfolds irreversibly, while SBD unfolds reversibly in the pH range 5.5-7.5 where aggregation does not occur. The unfolding of the catalytic domain of all glucoamylase forms seems to follow an irreversible one-step mechanism with no observable reversible intermediates on the experimental time scale. SBD of GA1 unfolds reversibly, and the ratio between the van't Hoff and calorimetric enthalpies is 1.4 ± 0.1 . Assignment of peaks of the DSC profile to the domains at pH 7.5 is achieved by using two different ligands: Acarbose, a very strong inhibitor that binds exclusively to the catalytic domain, and beta-cyclodextrin, a small starch analogue of which 2 molecules bind solely to the two binding sites present in SBD. Differences are seen in the unfolding processes of GA1 and GA2 since the former unfolds with one peak at all pH values, while the calorimetric trace of the latter can be resolved into more peaks depending on pH and the chemical composition of the buffers. In general, peaks corresponding to unfolding of GA2 are more complex than the peaks of GA1 and GACD. Some part of GA2 unfolds before the rest of the molecule which may correspond to the linker region or a particular early unfolding part of the catalytic domain. This leads to the conclusion that the structure of the GA2 molecule has a larger cooperative unfolding unit and is less stable than the structures of GA1 and GACD and that the C-terminal part of the linker region has a destabilizing effect on the catalytic domain.

Christensen T., Frandsen T. P., Kaarsholm N. C., Svensson B., and Sigurskjold B. W. (2002) Physicochemical characterisation of the two active site mutants Trp(52)-->Phe and Asp(55)-->Val of glucoamylase from *Aspergillus niger*. *Biochim Biophys Acta* **1601**, 163-171.

Abstract: Glucoamylase 1 (GA1) from *Aspergillus niger* is a multidomain starch hydrolysing enzyme that consists of a catalytic domain and a starch-binding domain connected by an O-glycosylated linker. The fungus also produces a truncated form without the starch-binding domain (GA2). The active site mutant Trp(52)-->Phe of both forms and the Asp(55)-->Val mutant of the GA1 form have been prepared and physicochemically characterised and compared to recombinant wild-type enzymes. The characterisation included substrate hydrolysis, inhibitor binding, denaturant stability, and thermal stability, and the consequences for the active site of glucoamylase are discussed. The circular dichroic (CD) spectra of the mutants were very similar to the wild-type enzymes, indicating that they have similar tertiary structures. The D55V GA1 mutant showed slower kinetics of hydrolysis of maltose and maltoheptaose with $\Delta \Delta G$ (double dagger) congruent with 22 kJ mol^{-1} , whereas the binding of the strong inhibitor acarbose was greatly diminished by $\Delta \Delta G$ degrees congruent with 52 kJ mol^{-1} . Both W52F mutant forms have almost the same stability as the wild-type enzyme, whereas the D55V GA1 mutant showed slight destabilisation both towards denaturant and heat (DSC). The difference between the CD unfolding curves recorded by near- and far-UV indicated that D55V GA1 unfolds through a molten globule intermediate.

Collins T., D'Amico S., Georlette D., Marx J. C., Huston A. L., and Feller G. (2006) A nondetergent sulfobetaine prevents protein aggregation in microcalorimetric studies. *Anal Biochem* **352**, 299-301.

Crawley S.W., de la Roche M.A., Lee S.F., Li Z., Chitayat S., Smith S.P., and Cote GP. (2006) Identification and characterization of an 8-kDa light chain associated with Dictyostelium discoideum MyoB, a class I myosin. *J Biol Chem*. **281**, 6307-15.

Abstract: Dictyostelium discoideum MyoB is a single-headed class I myosin. Analysis of purified MyoB by SDS-PAGE indicated the presence of an approximately 9-kDa light chain. A tryptic digest of MyoB yielded a partial sequence for the light chain that exactly matched a sequence in a 73-amino acid, 8,296-Da protein (dictyBase number DDB0188713). This protein, termed MlcB, contains two EF-hand motifs and shares approximately 30% sequence identity with the N- and C-terminal lobes of calmodulin. FLAG-MlcB expressed in Dictyostelium co-immunoprecipitated with MyoB but not with the related class myosins and MyoD. Recombinant MlcB bound Ca²⁺ with a K_d value of 0.2 microm and underwent a Ca²⁺-induced change in conformation that increased alpha-helical content and surface hydrophobicity. Mutational analysis showed that the first EF-hand was responsible for Ca²⁺ binding. In the presence and absence of Ca²⁺ MlcB was a monomer in solution and bound to a MyoB IQ motif peptide with a K_d value of approximately 0.5 microm. A MyoB head-neck construct with a Ser to Glu mutation at the TEDS site bound MlcB and displayed an actin-activated Mg²⁺ ATPase activity that was insensitive to Ca²⁺. We conclude that MlcB represents a novel type of small myosin light chain that binds to IQ motifs in a manner comparable with a single lobe of a typical four-EF-hand protein.

Creagh A. L., Tiong J. W., Tian M. M., Haynes C. A., and Jefferies W. A. (2005) Calorimetric studies of melanotransferrin (p97) and its interaction with iron. *J Biol Chem* **280**, 15735-15741.

Abstract: The mammalian molecule melanotransferrin (mTf), also called p97, is a member of the transferrin family of molecules. It exists in both secreted and glycosylphosphatidylinositol-anchored forms and is thought to play a role in angiogenesis and in transporting iron across the blood brain barrier. The binding affinity of iron to this molecule has not been formally established. Here, the binding of ferric ion (chelated with a 2-fold molar ratio of nitrilotriacetate) to mTf has been studied using isothermal titration calorimetry and differential scanning calorimetry. One iron-binding site was determined for mTf with similar binding characteristics to other transferrins. In the absence of bicarbonate, binding occurs quickly with an apparent association constant of 2.6×10^7 M⁻¹ at 25 degrees C. The presence of bicarbonate introduces kinetic effects that prevent direct determination of the apparent binding constant by isothermal titration calorimetry. Differential scanning calorimetry thermograms of mTf unfolding in the presence and absence of iron were therefore used to determine the apparent binding constant in the bicarbonate-containing system; at pH 7.5 and 25 degrees C, iron binding occurs in a 1:1 ratio with a K_(app) of 4.4×10^{17} M⁻¹. This affinity is intermediate between the high and low affinity lobes of transferrin and suggests that mTf is likely to play a significant role in iron transport where the high affinity lobe of transferrin is occupied or where transferrin is in proportionally low concentrations.

Creveld L. D., Meijberg W., Berendsen H. J., and Pepermans H. A. (2001) DSC studies of Fusarium solani pisi cutinase: consequences for stability in the presence of surfactants. *Biophys Chem* **92**, 65-75.

Abstract: The application of cutinase from Fusarium solani pisi as a fat-stain removing ingredient in laundry washing is hampered by its lack of stability in the presence of anionic surfactants. We postulate that the stability of cutinase towards anionics can be improved by mutations increasing its temperature stability. Thermal unfolding as measured with DSC, appears to be irreversible, though the thermograms are more symmetric than predicted by a simple irreversible model. In the presence of taurodeoxycholate (TDOC), the unfolding temperature is lower and the unfolding is reversible. We conclude that an early reversible unfolding intermediate exists in which a number of additional hydrophobic patches are exposed to the solvent, or preferentially are covered with TDOC. Improvement of the stability of cutinase with respect to both surfactants and thermal denaturation, should thus be directed toward the prevention of exposure of hydrophobic patches in the early intermediate.

Cseh R., Hetzer M., Wolf K., Kraus J., Bringmann G., and Benz R. (2000) Interaction of phloretin with membranes: on the mode of action of phloretin at the water-lipid interface. *Eur Biophys J* **29**, 172-183.

Abstract: The interaction of phloretin with single lipid bilayers on a spherical support and with multilamellar vesicles was studied by differential scanning calorimetry (DSC) and nuclear magnetic

resonance (NMR). The results indicated that phloretin interacts with the lipid layer and changes its structural parameters. In DSC experiments, phloretin in its neutral form strongly decreased the lipid phase transition temperature and slightly reduced the cooperativity of the phase transition within the lipid layer. In NMR measurements, phloretin led to an increase of the transverse relaxation time constant but had no effect on the spin-lattice relaxation time constant. The overall dipole moment of phloretin was experimentally determined and was found to be roughly 40% lower than has been published previously. This result suggested that the size of the dipole moment of phloretin does not provide such a high contribution to the effect of phloretin on the dipole potential of monolayers and bilayers as has been published previously. To understand the discrepancy between phloretin adsorption and dipole potential change, we performed computational conformational analysis of phloretin in the gas phase. The results showed that a wide distribution of the dipole moments of phloretin conformers exists, which mainly depends on the orientation of the OH moieties. The adsorption of phloretin as determined from its binding to solid supported bilayers differed from the one determined from dipole potential measurements on black lipid membranes. The difference between the phloretin dissociation constants of both types of experiments suggested a change of its dipole moment normal to the membrane surface in a concentration-dependent manner, which was in agreement with the results of the computational conformational analysis.

Dedova I. V., Nikolaeva O. P., Mikhailova V. V., dos Remedios C. G., and Levitsky D. I. (2004) Two opposite effects of cofilin on the thermal unfolding of F-actin: a differential scanning calorimetric study. *Biophys Chem* **110**, 119-128.

Abstract: Differential scanning calorimetry was used to examine the effects of cofilin on the thermal unfolding of actin. Stoichiometric binding increases the thermal stability of both G- and F-actin but at sub-saturating concentrations cofilin destabilizes F-actin. At actin:cofilin molar ratios of 1.5-6 the peaks corresponding to stabilized (66-67 degrees C) and destabilized (56-57 degrees C) F-actin are observed simultaneously in the same thermogram. Destabilizing effects of sub-saturating cofilin are highly cooperative and are observed at actin:cofilin molar ratios as low as 100:1. These effects are abolished by the addition of phalloidin or aluminum fluoride. Conversely, at saturating concentrations, cofilin prevents the stabilizing effects of phalloidin and aluminum fluoride on the F-actin thermal unfolding. These results suggest that cofilin stabilizes those actin subunits to which it directly binds, but destabilizes F-actin with a high cooperativity in neighboring cofilin-free regions.

den Blaauwen T., Fekkes P., de Wit J. G., Kuiper W., and Driessen A. J. (1996) Domain interactions of the peripheral preprotein Translocase subunit SecA. *Biochemistry* **35**, 11994-12004.

Abstract: The homodimeric SecA protein is the peripheral subunit of the preprotein translocase in bacteria. It binds the preprotein and promotes its translocation across the bacterial cytoplasmic membrane by nucleotide modulated coinserion and deinsertion into the membrane. SecA has two essential nucleotide binding sites (NBS; Mitchell & Oliver, 1993): The high-affinity NBS-I resides in the amino-terminal domain of the protein, and the low-affinity NBS-II is localized at 2/3 of the protein sequence. The nucleotide-bound states of soluble SecA were studied by site directed tryptophan fluorescence spectroscopy, tryptic digestion, differential scanning calorimetry, and dynamic light scattering. A nucleotide-induced conformational change of a carboxy-terminal domain of SecA was revealed by Trp fluorescence spectroscopy. The Trp fluorescence of a single Trp SecA mutant containing Trp775 decreased and increased upon the addition of NBS-I saturating concentrations of ADP or AMP-PNP, respectively. DSC measurements revealed that SecA unfolds as a two domain protein. Binding of ADP to NBS-I increased the interaction between the two domains whereas binding of AMP-PNP did not influence this interaction. When both NBS-I and NBS-II are bound by ADP, SecA seems to have a more compact globular conformation whereas binding of AMP-PNP seems to cause a more extended conformation. It is suggested that the compact ADP-bound conformation resembles the membrane deinserted state of SecA, while the more extended ATP-bound conformation may correspond to the membrane inserted form of SecA.

Dick S., Marrone L., Duewel H., Beecroft M., McCourt J., and Viswanatha T. (1999) Lysine: N6-hydroxylase: stability and interaction with ligands. *J Protein Chem* **18**, 893-903.

Abstract: Recombinant lysine:N6-hydroxylase, rLucD, which is isolated as an apoenzyme, requires FAD and NADPH for its catalytic function. rLucD preparations have been found to undergo time-dependent loss in monooxygenase function due to aggregation from the initial tetrameric state to a polytetrameric form(s),

a process which is reversible by treatment with thiols. Ligand-induced conformational changes in rIucD were assessed by monitoring its CD spectra, DSC profile, and susceptibility to both endo- as well as exopeptidases. The first two methods indicated the absence of any significant conformational change in rIucD, while the last approach revealed that FAD, and its analog ADP, can protect the protein from the deleterious action of proteases. NADPH was partially effective and L-lysine was ineffective in this regard. Deletion of the C-terminal segment, either by treatment with carboxypeptidase Y or by mutagenesis of iucD, results in the loss of rIucD's monooxygenase activity. These findings demonstrate the crucial role of the C-terminal segment in maintaining rIucD in its native conformation.

Dico A. S., Hancock J., Morrow M. R., Stewart J., Harris S., and Keough K. M. (1997) Pulmonary surfactant protein SP-B interacts similarly with dipalmitoylphosphatidylglycerol and dipalmitoylphosphatidylcholine in phosphatidylcholine/phosphatidylglycerol mixtures. *Biochemistry* **36**, 4172-4177.

Abstract: Porcine pulmonary surfactant-associated protein SP-B was incorporated into bilayers of chain-perdeuterated dipalmitoylphosphatidylglycerol (DPPG-d62) and into bilayers containing 70 mol % dipalmitoylphosphatidylcholine (DPPC) and 30 mol % DPPG-d62 or 70 mol % chain-perdeuterated DPPC (DPPC-d62) and 30 mol % DPPG. The effect of SP-B on the phase behavior, lipid chain order, and dynamics in these bilayers was examined using deuterium nuclear magnetic resonance (2H-NMR). In both DPPG-d62 and the mixed lipid system, SP-B is found to have little effect on chain order in the liquid crystalline phase. With 11% (w/w) SP-B present, both bilayer systems display a continuous change from liquid crystal to gel with no evidence of two-phase coexistence near the transition. Despite its limited effect on chain order in these bilayers, SP-B is found to strongly perturb chain deuteron transverse relaxation in the liquid crystal and gel phases of DPPG-d62 and the DPPC/DPPG (7:3) mixtures. The observation that SP-B associates with the bilayer in a way which substantially alters the slow motions responsible for transverse relaxation without significantly affecting chain order in either the liquid crystal or gel phases may place some constraints on possible models for that association.

Dignam J. D., Nada S., and Chaires J. B. (2003) Thermodynamic characterization of the binding of nucleotides to glycyl-tRNA synthetase. *Biochemistry* **42**, 5333-5340.

Abstract: The interaction of adenine nucleotides with glycyl-tRNA synthetase was examined by several experimental approaches. ATP and nonsubstrate ATP analogues render glycyl-tRNA synthetase more resistant to digestion by a number of proteases (thrombin, Arg-C, and chymotrypsin) at concentrations that correlate with their Michaelis constants or inhibition constants, consistent with their exerting an effect by binding at the ATP site. Glycine had little effect alone but potentiated the effect of ATP in increasing the resistance to thrombin digestion, consistent with the formation of an enzyme-bound adenylate. No protection from thrombin digestion was afforded by tRNA(gly). Binding constants were determined by isothermal titration calorimetry at 25 degrees C for ATP ($2.5 \times 10^5 \text{ M}^{-1}$), AMPPNP ($3.7 \times 10^5 \text{ M}^{-1}$), and AMPPCP ($2.2 \times 10^6 \text{ M}^{-1}$). The nucleotides had similar values for ΔH (-71 kJ mol^{-1}), with values for $T\Delta S$ that accounted for the differences in the binding constants. Near-ultraviolet CD spectra of the enzyme-nucleotide complexes indicate that the nucleotides are bound in the anti configuration. A glycyl-adenylate analogue, glycine sulfamoyl adenosine (GSAd), bound with a large value for ΔH (-187 kJ mol^{-1}), which was balanced by a large $T\Delta S$ term to give a binding constant ($3.7 \times 10^6 \text{ M}^{-1}$) only slightly larger than that of AMPPCP. Glycine binding to the enzyme could not be detected calorimetrically, and its presence did not change the thermodynamic parameters for binding of AMPPCP. AMPPNP and AMPPCP were not substrates for glycyl-tRNA synthetase. Analysis of the temperature dependence of ATP binding indicated that the heat capacity change is small, whereas the binding of GSAd is accompanied by a large negative heat capacity change ($-2.6 \text{ kJ K}^{-1} \text{ mol}^{-1}$). Titrations performed in buffers with different ionization enthalpies indicate that the large value for ΔH for the adenylate analogue does not arise from a coupled protonation event. Differential scanning calorimetry indicated that glycyl-tRNA synthetase is stabilized by nucleotides. Unfolding of the protein is irreversible, and thermodynamic parameters for unfolding could therefore not be determined. The results are consistent with a significant conformational transition in glycyl-tRNA synthetase coupled to the binding of GSAd.

Eckenhoff R. G., Tanner J. W., and Liebman P. A. (2001) Cooperative binding of inhaled anesthetics and ATP to firefly luciferase. *Proteins* **42**, 436-441.

Abstract: Firefly luciferase is considered a reasonable model of in vivo anesthetic targets despite being

destabilized by anesthetics, as reflected by differential scanning calorimetry (DSC). We examined the interaction between two inhaled anesthetics, ATP, luciferase, and temperature, using amide hydrogen exchange, tryptophan fluorescence, and photolabeling in an attempt to examine this apparent discrepancy. In the absence of ATP/Mg²⁺, halothane and bromoform cause destabilization, as measured by hydrogen exchange, suggesting nonspecific interactions. In the presence of ATP/Mg²⁺ and at room temperature, the anesthetics produce considerable stabilization with a negative ΔH , indicating population of a conformer with a specific anesthetic binding site. Stabilizing interactions are lost, however, at unfolding temperatures. We suggest that preferential binding to aggregated forms of luciferase explain the higher temperature destabilization detected with DSC. Our results demonstrate a cooperative binding equilibrium between native ligands and anesthetics, suggesting that similar interactions could underlie actions at biologically relevant targets.

Epanand R. F., Epanand R. M., and Jung C. Y. (1999) Glucose-induced thermal stabilization of the native conformation of GLUT 1. *Biochemistry* **38**, 454-458.

Abstract: The glucose transporter, GLUT 1, was purified from erythrocyte membranes and incorporated into vesicles of erythrocyte lipids. These protein-containing vesicles were studied with differential scanning calorimetry. It was found that the protein underwent an irreversible denaturation at 68.5 +/- 0.2 degreesC (at a scan rate of 0.25 degreesC/min) which was shifted to 72.6 +/- 0.2 degreesC in the presence of 500 mM D-glucose, while 500 mM L-glucose or 10 μ M cytochalasin B did not produce a significant shift. The calorimetric enthalpy was found to be 150 kcal/mol, independent of the presence of D-glucose. On a weight basis this value is lower than that for soluble proteins, but it is comparable to values obtained with other integral membrane proteins. The van't Hoff enthalpy is similar to the calorimetric enthalpy, within the experimental error, indicating that the transition is not likely to be cooperative. The activation energy is estimated from both the scan rate dependence of the transition temperature and from the shape of the DSC curve. The presence of 500 mM D-glucose slightly decreases the activation energy. It is concluded that the shift to a higher denaturation transition temperature in the presence of D-glucose is not a result of increased kinetic stability of GLUT 1.

Epanand R. F., Epanand R. M., and Jung C. Y. (2001) Ligand-modulation of the stability of the glucose transporter GLUT 1. *Protein Sci* **10**, 1363-1369.

Abstract: The glucose transporter GLUT 1 was isolated from human erythrocytes and reconstituted into endogenous membrane lipids. Results from thermal denaturation studies, using differential scanning calorimetry, indicate that the thermal denaturation temperature of GLUT 1 is significantly lower in the presence of ATP. The lowering of this transition temperature is very dependent on pH. At more acidic pH, ATP has a greater effect of lowering the thermal denaturation temperature of the protein. For example, with 4.8 mM ATP, the denaturation endotherm is lowered by over 10 degrees at pH 4.3, whereas at pH 7.4, ATP does not alter this transition temperature. However, a change in pH alone, in the absence of ATP, has very little effect on the denaturation temperature. Both glucose and salt partially reverse the lowering of the temperature of thermal denaturation caused by ATP. Studies of acrylamide quenching of the Trp residues of GLUT 1 indicate that at neutral pH, ATP increases the Stern-Volmer quenching constant, while glucose lowers it. The results indicate that ATP binds to GLUT 1 and destabilizes the native structure, leading to a lowering of the thermal denaturation temperature and an increase in acrylamide quenching. The effects of ATP are reversed in part by glucose and are also partly electrostatic in nature.

Franco R., Bai G., Prosiniecki V., Abrunhosa F., Ferreira G. C., and Bastos M. (2005) Porphyrin-substrate binding to murine ferrochelatase: effect on the thermal stability of the enzyme. *Biochem J* **386**, 599-605.

Abstract: Ferrochelatase (EC 4.99.1.1), the terminal enzyme of the haem biosynthetic pathway, catalyses the chelation of Fe(II) into the protoporphyrin IX ring. The energetics of the binding between murine ferrochelatase and mesoporphyrin were determined using isothermal titration calorimetry, which revealed a stoichiometry of one molecule of mesoporphyrin bound per protein monomer. The binding is strongly exothermic, with a large intrinsic enthalpy ($\Delta H = -97.1$ kJ x mol⁻¹), and is associated with the uptake of two protons from the buffer. This proton transfer suggests that hydrogen bonding between ferrochelatase and mesoporphyrin is a key factor in the thermodynamics of the binding reaction. Differential scanning calorimetry thermograms indicated a co-operative two-state denaturation process with a single transition temperature of 56 degrees C for wild-type murine ferrochelatase. An increase in the thermal stability of ferrochelatase is dependent upon mesoporphyrin binding. Similarly, murine ferrochelatase variants, in

which the active site Glu-289 was replaced by either glutamine or alanine and, when purified, contained specifically-bound protoporphyrin, exhibited enhanced protein stability when compared with wild-type ferrochelatase. However, in contrast with the wild-type enzyme, the thermal denaturation of ferrochelatase variants was best described as a non-co-operative denaturation process.

Galan A., Llorca O., Valpuesta J. M., Perez-Perez J., Carrascosa J. L., Menendez M., Banuelos S., and Muga A. (1999) ATP hydrolysis induces an intermediate conformational state in GroEL. *Eur J Biochem* **259**, 347-355.

Abstract: The conformational properties of the molecular chaperone GroEL in the presence of ATP, its non-hydrolyzable analog 5'-adenylymidodiphosphate (AMP-PNP), and ADP have been analyzed by differential scanning calorimetry (DSC), Fourier-transform infra-red (FT-IR) and fluorescence spectroscopy. Nucleotide binding to one ring promotes a decrease in the T_m value of the GroEL thermal transition that is reversed when both rings are filled with nucleotide, indicating that the sequential occupation of the two protein rings by these nucleotides has different effects on the GroEL thermal denaturation process. In addition, ATP induces a conformational change in GroEL characterized by (a) the appearance of a reversible low temperature endotherm in the DSC profiles of the protein, and (b) an enhanced binding of the hydrophobic probe 8-anilino-naphthalene-1-sulfonate (ANS), which strictly depends on ATP hydrolysis. The similar sensitivity to K^+ of the temperature range where activation of the GroEL ATPase activity, the low temperature endotherm, and the increase of the ANS fluorescence are observed strongly indicates the existence of a conformational state of GroEL during ATP hydrolysis, different from that generated on ADP or AMP-PNP binding. To achieve this intermediate conformation, GroEL mainly modifies its tertiary and quaternary structures, leading to an increased exposure of hydrophobic surfaces, with minor rearrangements of its secondary structure.

Garnier C., Lafitte D., Tsvetkov P. O., Barbier P., Leclerc-Devin J., Millot J. M., Briand C., Makarov A. A., Catelli M. G., and Peyrot V. (2002) Binding of ATP to heat shock protein 90: evidence for an ATP-binding site in the C-terminal domain. *J Biol Chem* **277**, 12208-12214.

Abstract: The presence of a nucleotide binding site on hsp90 was very controversial until x-ray structure of the hsp90 N-terminal domain, showing a nonconventional nucleotide binding site, appeared. A recent study suggested that the hsp90 C-terminal domain also binds ATP (Marcu, M. G., Chadli, A., Bouhouche, I., Catelli, M. G., and Neckers, L. M. (2000) *J. Biol. Chem.* **275**, 37181-37186). In this paper, the interactions of ATP with native hsp90 and its recombinant N-terminal (positions 1-221) and C-terminal (positions 446-728) domains were studied by isothermal titration calorimetry, scanning differential calorimetry, and fluorescence spectroscopy. Results clearly demonstrate that hsp90 possesses a second ATP-binding site located on the C-terminal part of the protein. The association constant between this domain of hsp90 and ATP-Mg and a comparison with the binding constant on the full-length protein are reported for the first time. Secondary structure prediction revealed motifs compatible with a Rossmann fold in the C-terminal part of hsp90. It is proposed that this potential Rossmann fold may constitute the C-terminal ATP-binding site. This work also suggests allosteric interaction between N- and C-terminal domains of hsp90.

Golitsina N. L., Bobkov A. A., Dedova I. V., Pavlov D. A., Nikolaeva O. P., Orlov V. N., and Levitsky D. I. (1996) Differential scanning calorimetric study of the complexes of modified myosin subfragment 1 with ADP and vanadate or beryllium fluoride. *J Muscle Res Cell Motil* **17**, 475-485.

Abstract: The effects of various modifications of rabbit skeletal myosin subfragment 1 on the thermal denaturation of subfragment 1 in ternary complexes with Mg-ADP and orthovanadate (V1) or beryllium fluoride (BeFx) have been studied by differential scanning calorimetry. It has been shown that specific modifications of SH1 group of Cys-707 by different sulfhydryl reagents, trinitrophenylation of Lys-83, and reductive methylation of lysine residues promote the decomposition of the S1.ADP.Vi complex and change the character of structural transitions of the subfragment 1 molecule induced by the formation of this complex, but they have much less or no influence on subfragment 1 thermal stability in the S1.ADP.BeFx complex. Thus, the differential scanning calorimetric studies on modified subfragment 1 preparations reveal a significant difference between S1.ADP.Vi and S1.ADP.BeFx complexes. It is suggested that S1.ADP.Vi and S1.ADP.BeFx complexes represent structural analogues of different transition states of the ATPase cycle, namely the intermediate states $S1^{**}.ADP.Pi$ and $S1^*.ATP$, respectively. It is also proposed that during formation of the S1.ADP.Vi complex the region containing both Cys-707 and Lys-83 plays an

important role in the spread of conformational changes from the active site of subfragment 1 ATPase throughout the structure of the entire subfragment 1 molecule. In such a case, the effects of reductive methylation of lysine residues on the subfragment 1 structure in the S1.ADP.Vi complex are related to the modification of Lys-83.

Gordo S., Martos V., Santos E., Menendez M., Bo C., Giralt E. and de M. J. (2008) Stability and structural recovery of the tetramerization domain of p53-R337H mutant induced by a designed templating ligand. *Proc. Natl. Acad. Sci U. S. A* **105**, 16426-16431.

Abstract: Protein p53 is a transcription factor crucial for cell cycle and genome integrity. It is able to induce both cell arrest when DNA is damaged and the expression of DNA repair machinery. When the damage is irreversible, it triggers apoptosis. Indeed, the protein, which is a homotetramer, is mutated in most human cancers. For instance, the inherited mutation p53-R337H results in destabilization of the tetramer and, consequently, leads to an organism prone to tumor setup. We describe herein a rational designed molecule capable of holding together the four monomers of the mutated p53-R337H protein, recovering the tetramer integrity as in the wild-type structure. Two ligand molecules, based on a conical calix[4]arene with four cationic guanidiniomethyl groups at the wider edge (upper rim) and hydrophobic loops at the narrower edge (lower rim), fit nicely and cooperatively into the hydrophobic clefts between two of the monomers at each side of the protein and keep the tetrameric structure, like molecular templates, by both ion-pair and hydrophobic interactions. We found a good agreement between the structure of the complex and the nature of the interactions involved by a combination of theory (molecular dynamics) and experiments (circular dichroism, differential scanning calorimetry and ^1H saturation transfer difference NMR)

Guzman-Casado M., Cardenete A., Gimenez-Gallego G., and Parody-Morreale A. (2001) Myo-inositol hexasulphate and low molecular weight heparin binding to human acidic fibroblast growth factor: a calorimetric and FTIR study. *Int J Biol Macromol* **28**, 305-313.

Abstract: The interaction of an amino-terminal-truncated 139 amino-acids form of human acidic fibroblast growth factor with myo-inositol hexasulphate and low molecular weight (3500 g mol^{-1}) heparin has been studied by isothermal titration calorimetry, differential scanning calorimetry and Fourier transform infrared spectroscopy. A slightly higher affinity for the monosaccharide has been measured. The binding of the ligands causes an increase of 13--15 degrees C in the melting temperature of the free protein (45 degrees C). From measured enthalpy and heat capacity changes, calculations of changes in accessible surface areas have been made. These calculations, together with infrared spectroscopy data, indicate that a small conformational change is induced by the binding of both ligands. This conformational change would affect the tertiary structure, not the secondary one.

Haun M. F., Wirth M., and Ruterjans H. (1995) Calorimetric investigation of thermal stability and ligand-binding characteristics of disulfide-bond-cleaved ribonuclease T1. *Eur J Biochem* **227**, 516-523.

Abstract: A combination of differential titration calorimetry and differential scanning calorimetry was used to study the effect of disulfide bond cleavage and reaction with iodoacetamide of ribonuclease T1 on both the binding of nucleotides and the thermal stability of the free enzyme species. Although guanosine monophosphates still bind to the active site of the modified protein the transition temperature of unfolding and the transition enthalpy decrease drastically indicating a relatively loose structure. The calorimetric data presented in this study suggest a cooperative linkage between the site of the disulfide bonds, the ligand-binding site, and the general thermodynamic stability of the enzyme.

Hianik T., Ponikova S., Bagel'ova J., and Antalík M. (2006) Specific volume and compressibility of human serum albumin-polyanion complexes. *Bioorg Med Chem Lett* **16**, 274-279.

Abstract: The ultrasound velocimetry, densitometry, and differential scanning calorimetry have been used to study the formation of the complexes between human serum albumin (HSA) and polyanions heparin (HEP) and/or dextran sulfate (DS). The values of the ultrasound velocity and specific volume allowed us to determine the specific adiabatic compressibility, $\phi(K)/\beta(0)$, which reflects the degree of volume compressibility of the complexes. We showed that in the presence of HEP and DS the adiabatic compressibility of HSA decreases with increasing concentration of polyanions. HEP more strongly interacts with HSA than DS. pH of electrolyte in the range 4.7-8.5 weakly affects the adiabatic compressibility. Changes of compressibility of HSA can be caused by increase of the hydration due to the

formation of the HSA-polyanion complexes and due to partial unfolding of HSA. The HSA-polyanion interaction resulted in decrease of phase transition temperature of the protein. This evidences about protein destabilization in the presence of polyanions.

Huus K., Havelund S., Olsen H. B., Sigurskjold B. W., van de W. M., and Frokjaer S. (2006) Ligand binding and thermostability of different allosteric states of the insulin zinc-hexamer. *Biochemistry* **45**, 4014-4024.

Abstract: The influence of ligand binding and conformation state on the thermostability of hexameric zinc-insulin was studied by differential scanning calorimetry (DSC). The insulin hexamer exists in equilibrium between the forms T6, T3R3, and R6. Phenolic ligands induce and stabilize the T3R3- and R6-states which are further stabilized by binding of certain anions that do not stabilize the T6-state. It was shown that the thermostability of the resorcinol-stabilized R6-state was significantly higher than that of the T6-state. Further analysis showed that phenol- and m-cresol-stabilized R6-hexamer loses three ligands before reaching the unfolding temperature and hence unfolds from the T3R3-state. The relative affinity of the four tested anionic ligands was found, by DSC, to be thiocyanate > or = 4-hydroxy-3-nitrobenzoate >> p-aminobenzoate >> chloride. The results correlate with other methods and demonstrate that DSC provides a general and useful method of evaluation of both phenolic and anionic ligand binding to insulin without the use of probes or other alterations of the system of interest. However, it is a prerequisite that the binding is strong enough to saturate the binding sites at temperatures around the unfolding transition.

Jones C. L., Fish F., and Muccio D. D. (2002) Determination of RNase A/2'-cytidine monophosphate binding affinity and enthalpy by a global fit of thermal unfolding curves. *Anal Biochem* **302**, 184-190.

Abstract: A spectropolarimeter was used to measure the thermal response curves of RNase A in the presence and absence of the ligand cytidine 2'-monophosphate. A coupled equilibrium model was used to describe the dissociation of the protein-ligand complex ($NL \rightleftharpoons N + L$) and the thermal unfolding of the free protein ($N \rightleftharpoons U$). The unfolding curves of the protein in the presence of several different concentrations of ligand were fit to this coupled equilibrium model using global linkage analysis. The best-fitted values for the thermal unfolding of the apo-protein were 60.9 +/- 0.2 degrees C (T_m) and 105.5 +/- 1.4 kcal/mol (ΔH), while the fitted values for the dissociation of the protein-ligand complex were 1.6 +/- 0.4 μM ($K(D)$) and 18.7 +/- 1.0 kcal mol⁻¹ ($\Delta H(L)$). These values were in excellent agreement with values obtained by other methods. The sensitivity of the data fitting was compared using linear or quadratic temperature dependence for the response curves of the free ligand (L), native apo-protein (N), native ligand-bound protein (NL), and unfolded apo-protein (U). There was no significant improvement in the precision of the fitted data when the temperature-dependent response for each species (N, L, NL, and U) was expressed as quadratic functions of temperature.

Jones L. S., Peek L. J., Power J., Markham A., Yazzie B., and Middaugh C. R. (2005) Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens. *J Biol Chem* **280**, 13406-13414.

Abstract: The effect of adsorption onto aluminum salt adjuvants on the structure and stability of three model protein antigens was studied using fluorescence and Fourier transform infrared spectroscopies, as well as isothermal titration and differential scanning calorimetric techniques. Lysozyme was preferentially adsorbed to aluminum phosphate (Adju-Phos), whereas ovalbumin and bovine serum albumin were better adsorbed to aluminum hydroxide (Alhydrogel). A linearized Langmuir adsorption isotherm was used to obtain information regarding the binding interactions between proteins and adjuvants. Binding energetics and stoichiometry data obtained from isothermal titration calorimetry measurements were complex. Based on the spectroscopic and differential scanning calorimetry studies, the structure of all three proteins, when adsorbed to the surface of an aluminum salt, was altered in such a way as to render the proteins less thermally stable. Besides the pharmaceutical significance of this destabilization, we consider the possibility that this phenomenon may facilitate the presentation of antigens and thus contribute to the adjuvant activity of the aluminum salts.

Kahmann J. D., O'Brien R., Werner J. M., Heinegard D., Ladbury J. E., Campbell I. D., and Day A. J. (2000) Localization and characterization of the hyaluronan-binding site on the link module from human TSG-6. *Structure Fold Des* **8**, 763-774.

Abstract: BACKGROUND: The interactions of hyaluronan (HA) with proteins are important in extracellular matrix integrity and leukocyte migration and are usually mediated by a domain termed a Link module. Although the tertiary structure of a Link module has been determined, the molecular basis of HA-protein interactions remains poorly understood. RESULTS: Isothermal titration calorimetry was used to characterize the interaction of the Link module from human TSG-6 (Link_TSG6) with HA oligosaccharides of defined length (HA(4)-HA(16)). All oligomers bound (except HA(4)) with $K(d)$ values ranging from 0.2-0.5 μ M at 25 degrees C. The reaction is exothermic with a favourable entropy and the thermodynamic profile is similar to those of other glycosaminoglycan-protein interactions. The HA(8) recognition site on Link_TSG6 was localized by comparing nuclear magnetic resonance (NMR) spectra from a 1:1 complex with free protein. Residues perturbed on HA binding include both amino acids that are likely to be directly involved in the interaction (i.e., Lys11, Tyr59, Asn67, Phe70, Lys72 and Tyr78) and those affected by a ligand-induced conformational change in the beta4/beta5 loop. The sidechain of Asn67 becomes more rigid in the complex suggesting that it is in close proximity to the binding site. CONCLUSIONS: In TSG-6 a single Link module is sufficient for a high-affinity interaction with HA. The HA-binding surface on Link_TSG6 is found in a similar position to that suggested previously for CD44, indicating that its location might be conserved across the Link module superfamily. Here we find no evidence for the involvement of linear sequence motifs in HA binding.

Kedracka-Krok S. and Wasylewski Z. (2003) A differential scanning calorimetry study of tetracycline repressor. *Eur J Biochem* **270**, 4564-4573.

Abstract: Tetracycline repressor (TetR), which constitutes the most common mechanism of bacterial resistance to an antibiotic, is a homodimeric protein composed of two identical subunits, each of which contains a domain possessing a helix-turn-helix motif and a domain responsible for binding tetracycline. Binding of tetracycline in the protein pocket is accompanied by conformational changes in TetR, which abolish the specific interaction between the protein and DNA. Differential scanning calorimetry (DSC) and CD measurements, performed at pH 8.0, were used to observe the thermal denaturation of TetR in the absence and presence of tetracycline. The DSC results show that, in the absence of tetracycline, the thermally induced transitions of TetR can be described as an irreversible process, strongly dependent on scan rate and indicating that the protein denaturation is under kinetic control described by the simple kinetic scheme: $N(2) \rightarrow D(2)$, where k is a first-order kinetic constant, N is the native state, and D is the denatured state. On the other hand, analysis of the scan rate effect on the transitions of TetR in the presence of tetracycline shows that thermal unfolding of the protein can be described by the two-state model: $N(2) \rightarrow U(2) \rightarrow D$. In the proposed model, TetR in the presence of tetracycline undergoes co-operative unfolding, characterized by an enthalpy change ($\Delta H(\text{cal}) = 1067 \text{ kJ} \times \text{mol}^{-1}$) and an entropy change ($\Delta S = 3.1 \text{ kJ} \times \text{mol}^{-1}$).

Kocisova E., Jancura D., Sanchez-Cortes S., Miskovsky P., Chinsky L., and Garcia-Ramos J. V. (1999) Interaction of antiviral and antitumor photoactive drug hypocrellin A with human serum albumin. *J Biomol Struct Dyn* **17**, 111-120.

Abstract: Absorption, resonance Raman, surface-enhanced Raman spectroscopy and differential scanning microcalorimetry were employed to study the interaction of hypocrellin A with human serum albumin. The identification of the binding place for hypocrellin A as well as the model for the albumin-hypocrellin A complex are proposed. In this model hypocrellin A interacts with albumin through more than one binding site placed on the protein surface. This model of non-specific interaction could explain why the absorption spectrum of hypocrellin A does not change in the presence of albumin and why the presence of the drug does not change significantly the thermodynamic parameters of the protein, while the Raman spectra show evident changes concerning both the protein and the drug structure. Even if hypocrellin A does not interact with an interior binding site, it can affect deeply the general albumin structure.

Kristl S., Zhao S., Falsone S. F., Somerville R. L., and Kungl A. J. (2001) The influence of ATP on the association and unfolding of the tyrosine repressor ligand response domain of Haemophilus influenzae. *Biochem Biophys Res Commun* **280**, 81-84.

Abstract: The secondary structure of the ligand response domain of the Haemophilus influenzae tyrosine repressor, TyrR(lrd), was investigated using CD spectroscopy which revealed 42.5% alpha-helix, 17.6% beta-sheet, and 39.9% loops. Quaternary structure analysis by fluorescence anisotropy showed that TyrR(lrd) is monomeric at a concentration of 100 nM to 2 μ M but that the protein readily dimerizes in the

presence of its natural ligand ATP. Equilibrium unfolding studies of TyrR(Ird) using guanidinium hydrochloride suggested a two-state model with no detectable stable intermediates. The unfolding transition monitored by CD spectroscopy was responsive to tyrosine and ATP resulting in a shift to higher denaturant concentrations in the presence of these ligands. Differential scanning calorimetry yielded melting temperatures, T_m , of 51.15 and 58.07 degrees C for the unliganded and for the ATP-liganded protein, respectively. ATP is thus proposed to be a major structural cofactor for the molecular architecture of TyrR(Ird).

Kroe R. R., Regan J., Proto A., Peet G. W., Roy T., Landro L. D., Fuschetto N. G., Pargellis C. A., and Ingraham R. H. (2003) Thermal denaturation: a method to rank slow binding, high-affinity P38alpha MAP kinase inhibitors. *J Med Chem* **46**, 4669-4675.

Abstract: It has been reported that the diaryl urea class of p38alpha inhibitors binds to p38 map kinase with both high affinity and slow binding kinetics (Pargellis et al. *Nat. Struct. Biol.* 2002, 9, 268-272). The slow binding kinetics of this class of inhibitors is believed to be the result of binding to an allosteric pocket adjacent to the p38alpha active site. The use of traditional kinetic and equilibrium methods to measure the binding affinity of this class of compounds has created many challenges for determination of structure-activity relationships (SAR). The thermal denaturation method provides a means of measuring high-affinity interactions. In this paper, the method of thermal denaturation will be described as it has been applied to the diaryl urea class of p38 map kinase inhibitors.

Krupakar J., Swaminathan C. P., Das P. K., Surolia A., and Podder S. K. (1999) Calorimetric studies on the stability of the ribosome-inactivating protein abrin II: effects of pH and ligand binding. *Biochem J* **338** (Pt 2), 273-279.

Abstract: The effects of pH and ligand binding on the stability of abrin II, a heterodimeric ribosome-inactivating protein, and its subunits have been studied using high-sensitivity differential scanning calorimetry. At pH7.2, the calorimetric scan consists of two transitions, which correspond to the B-subunit [transition temperature (T_m) 319.2K] and the A-subunit (T_m 324.6K) of abrin II, as also confirmed by studies on the isolated A-subunit. The calorimetric enthalpy of the isolated A-subunit of abrin II is similar to that of the higher-temperature transition. However, its T_m is 2.4K lower than that of the higher-temperature peak of intact abrin II. This indicates that there is some interaction between the two subunits. Abrin II displays increased stability as the pH is decreased to 4.5. Lactose increases the T_m values as well as the enthalpies of both transitions. This effect is more pronounced at pH7.2 than at pH4.5. This suggests that ligand binding stabilizes the native conformation of abrin II. Analysis of the B-subunit transition temperature as a function of lactose concentration suggests that two lactose molecules bind to one molecule of abrin II at pH7.2. The presence of two binding sites for lactose on the abrin II molecule is also indicated by isothermal titration calorimetry. Plotting ΔH_m (the molar transition enthalpy at T_m) against T_m yielded values for ΔC_p (change in excess heat capacity) of 27 ± 2 kJ.mol⁻¹.K⁻¹ for the B-subunit and 20 ± 1 kJ.mol⁻¹.K⁻¹ for the A-subunit. These values have been used to calculate the thermal stability of abrin II and to surmise the mechanism of its transmembrane translocation.

Kurganov B. I., Kornilaev B. A., Chebotareva N. A., Malikov V. P., Orlov V. N., Lyubarev A. E., and Livanova N. B. (2000) Dissociative mechanism of thermal denaturation of rabbit skeletal muscle glycogen phosphorylase b. *Biochemistry* **39**, 13144-13152.

Abstract: The thermal stability of rabbit skeletal muscle glycogen phosphorylase b was characterized using enzymological inactivation studies, differential scanning calorimetry, and analytical ultracentrifugation. The results suggest that denaturation proceeds by the dissociative mechanism, i.e., it includes the step of reversible dissociation of the active dimer into inactive monomers and the following step of irreversible denaturation of the monomer. It was shown that glucose 1-phosphate (substrate), glucose (competitive inhibitor), AMP (allosteric activator), FMN, and glucose 6-phosphate (allosteric inhibitors) had a protective effect. Calorimetric study demonstrates that the cofactor of glycogen phosphorylase-pyridoxal 5'-phosphate-stabilizes the enzyme molecule. Partial reactivation of glycogen phosphorylase b preheated at 53 degrees C occurs after cooling of the enzyme solution to 30 degrees C. The fact that the rate of reactivation decreases with dilution of the enzyme solution indicates association of inactive monomers into active dimers during renaturation. The allosteric inhibitor FMN enhances the rate of phosphorylase b reactivation.

Lafitte D., Lamour V., Tsvetkov P. O., Makarov A. A., Klich M., Deprez P., Moras D., Briand C., and Gilli R. (2002) DNA gyrase interaction with coumarin-based inhibitors: the role of the hydroxybenzoate isopentenyl moiety and the 5'-methyl group of the noviose. *Biochemistry* **41**, 7217-7223.

Abstract: DNA gyrase is a major bacterial protein that is involved in replication and transcription and catalyzes the negative supercoiling of bacterial circular DNA. DNA gyrase is a known target for antibacterial agents since its blocking induces bacterial death. Quinolones, coumarins, and cyclothialidines have been designed to inhibit gyrase. Significant improvements can still be envisioned for a better coumarin-gyrase interaction. In this work, we obtained the crystal costructures of the natural coumarin clorobiocin and a synthetic analogue with the 24 kDa gyrase fragment. We used isothermal titration microcalorimetry and differential scanning calorimetry to obtain the thermodynamic parameters representative of the molecular interactions occurring during the binding process between coumarins and the 24 kDa gyrase fragment. We provide the first experimental evidence that clorobiocin binds gyrase with a stronger affinity than novobiocin. We also demonstrate the crucial role of both the hydroxybenzoate isopentenyl moiety and the 5'-alkyl group on the noviose of the coumarins in the binding affinity for gyrase.

Lamb H. K., Leslie K., Dodds A. L., Nutley M., Cooper A., Johnson C., Thompson P., Stammers D. K., and Hawkins A. R. (2003) The negative transcriptional regulator NmrA discriminates between oxidized and reduced dinucleotides. *J Biol Chem* **278**, 32107-32114.

Abstract: NmrA, a transcription repressor involved in the regulation of nitrogen metabolism in *Aspergillus nidulans*, is a member of the short-chain dehydrogenase reductase superfamily. Isothermal titration calorimetry and differential scanning calorimetry have been used to show NmrA binds NAD⁺ and NADP⁺ with similar affinity (average KD 65 μ M) but has a greatly reduced affinity for NADH and NADPH (average KD 6.0 mM). The structure of NmrA in a complex with NADP⁺ reveals how repositioning a His-37 side chain allows the different conformations of NAD⁺ and NADP⁺ to be accommodated. Modeling NAD(P)H into NmrA indicated that steric clashes, attenuation of electrostatic interactions, and loss of aromatic ring stacking can explain the differing affinities of NAD(P)⁺/NAD(P)H. The ability of NmrA to discriminate between the oxidized and reduced forms of the dinucleotides may be linked to a possible role in redox sensing. Isothermal titration calorimetry demonstrated that NmrA and a C-terminal fragment of the GATA transcription factor AreA interacted with a 1:1 stoichiometry and an apparent KD of 0.26 μ M. NmrA was unable to bind the nitrogen metabolite repression signaling molecules ammonium or glutamine.

Levashov P., Orlov V., Boschi-Muller S., Talfournier F., Asryants R., Bulatnikov I., Muronetz V., Branlant G., and Nagradova N. (1999) Thermal unfolding of phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase studied by differential scanning calorimetry. *Biochim Biophys Acta* **1433**, 294-306.

Abstract: Thermal unfolding parameters were determined for a two-domain tetrameric enzyme, phosphorylating D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and for its isolated NAD(+)-binding domain. At pH 8.0, the transition temperatures ($t(\max)$) for the apoforms of the native *Bacillus stearothermophilus* GAPDH and the isolated domain were 78.3 degrees C and 61.9 degrees C, with calorimetric enthalpies ($\Delta H(\text{cal})$) of 4415 and 437 kJ/mol (or 30.7 and 22.1 J/g), respectively. In the presence of nearly saturating NAD(+) concentrations, the $t(\max)$ and the $\Delta H(\text{cal})$ increased by 13.6 degrees C and by 2365 kJ/mol, respectively, for the native apoenzyme, and by 2.8 degrees C and 109 kJ/mol for the isolated domain. These results indicate that interdomain interactions are essential for NAD(+) to produce its stabilizing effect on the structure of the native enzyme. The thermal stability of the isolated NAD(+)-binding domain increased considerably upon transition from pH 6.0 to 8.0. By contrast, native GAPDH exhibited greater stability at pH 6.0; similar pH-dependencies of thermal stability were displayed by GAPDHs isolated from rabbit muscle and *Escherichia coli*. The binding of NAD(+) to rabbit muscle apoenzyme increased $t(\max)$ and $\Delta H(\text{cal})$ and diminished the widths of the DSC curves; the effect was found to grow progressively with increasing coenzyme concentrations. Alkylation of the essential Cys149 with iodoacetamide destabilized the apoenzyme and altered the effect of NAD(+). Replacement of Cys149 by Ser or by Ala in the *B. stearothermophilus* GAPDH produced some stabilization, the effect of added NAD(+) being basically similar to that observed with the wild-type enzyme. These data indicate that neither the ion pairing between Cys149 and His176 nor the charge transfer interaction between Cys149 and NAD(+) make any significant contribution to the stabilization of the enzyme's native tertiary structure and the accomplishment of NAD(+)-induced conformational changes. The H176N mutant exhibited dramatically lower heat stability, as reflected in the values of both $\Delta H(\text{cal})$ and $t(\max)$. Interestingly,

NAD(+) binding resulted in much wider heat capacity curves, suggesting diminished cooperativity of the unfolding transition.

Lin C. T., Chyan Y. G., Kresheck G. C., Bitting H. C., Jr., and el Sayed M. A. (1989) Interaction of dibucaine.HCl local anesthetics with bacteriorhodopsin in purple membrane: a spectroscopic study. *Photochem Photobiol* **49**, 641-648.

Abstract: Several spectroscopic techniques (absorption, emission, transient absorption and differential scanning calorimetry--DSC) were used to investigate the deprotonation of dibucaine.HCl in a hydrophobic environment, and the interaction sites and mechanisms of the local anesthetic dibucaine.HCl on bacteriorhodopsin (bR) in purple membrane. The important results are summarized as follows: (1) the visible absorption features of native ($\lambda_{\text{max}} = 568 \text{ nm}$) and deionized ($\lambda_{\text{max}} = 608 \text{ nm}$) bR are sensitive to the amount of dibucaine.HCl added; (2) the emission spectrum of dibucaine.HCl embedded in the retinal-free mutant bR is similar to that of dibucaine free base in Triton X-100 micellar solutions; (3) the phosphorescence emission of dibucaine at 77 K is completely quenched by bR and the fluorescence quenching rate for the incorporated dibucaine.HCl in bR was determined as $k_q = 4.09 \times 10^{13} \text{ M}^{-1} \text{ s}^{-1}$; (4) the incorporation of dibucaine.HCl in bR inhibits the slow component rate of formation of M412 and decreases the amount of M412 formation in the photochemical cycle of bR; and (5) the thermal stability of native bR was measured by DSC in the presence and absence of dibucaine and yielded an endothermic transition at 95.9 ± 1.0 degrees C with 13.6 J/g ($3.25 \pm 0.12 \text{ cal/g}$) of enthalpy changes. All observations suggest that the action site of the local anesthetic, dibucaine.HCl, is near or at the chromophore, i.e. the retinal Schiff base of bR. The anesthetic action on bR purple membrane is probably via a specific site binding, but not a conformational mechanism.

Lohner K., Sen A. C., Pranker R., Esser A. F., and Perrin J. H. (1994) Effects of drug-binding on the thermal denaturation of human serum albumin. *J Pharm Biomed Anal* **12**, 1501-1505.

Abstract: Asymmetric thermograms of defatted albumin, alone and in the presence of two model drugs, have been obtained in phosphate buffers at three pH values. The albumin is less thermally stable in the N form, but is protected by both drugs. The nonsteroidal antiinflammatory benoxaprofen offers more protection than warfarin against thermal denaturation.

Lorinczy D. and Belagyi J. (1995) Effects of nucleotide on skeletal muscle myosin unfolding in myofibrils by DSC. *Biochem Biophys Res Commun* **217**, 592-598.

Abstract: The thermal unfolding of myosin in skeletal muscle myofibrils was studied by differential scanning calorimetry (DSC). In the absence of nucleotide two major transitions with T_m of 52 degrees C and 58 degrees C, and a minor transition with T_m of 19 degrees C were detected. The unfolding can be characterized with a total enthalpy of $-90 \pm 6.1 \text{ mJ/g}$ protein. The major transition with T_m of 58 degrees C was independent of the presence of nucleotide and orthovanadate (Vi), and it can be assigned to the unfolding of the alpha-helical rod part of myosin and partly to actin. In the presence of MgADP, the minor transition shifted to higher temperature, indicating changes between the heavy chain of subfragment-1 and the LC-2 light chain. The transition with T_m of 52 degrees C exhibited a significant broadening and a small shift to lower temperature. It indicates an internal domain or segmental rearrangement of the myosin motor. Upon addition of MgADP and Vi , a shift to higher temperature was observed for the lower major transition, evidencing that with trapped ADP and Vi the intermolecular interactions stabilized the myosin head region.

Martinez J. C., El Harrou M., Filimonov V. V., Mateo P. L., and Fersht A. R. (1994) A calorimetric study of the thermal stability of barnase and its interaction with 3'GMP. *Biochemistry* **33**, 3919-3926.

Abstract: We have used high-sensitivity differential scanning calorimetry to characterize the thermal stability of barnase from *Bacillus amyloliquefaciens* in the pH range 2.0-5.0. The energetics of the interaction between barnase and its inhibitor 3'GMP have been studied by isothermal titration calorimetry in the temperature range 15-30 degrees C. Scanning calorimetry experiments were also made with the protein in the presence of various concentrations of 3'GMP at pH 4.5. A novel, simple procedure is proposed to obtain binding parameters from scanning calorimetry data. This method is based on the calculation of the partition functions of the free and the ligand-bound protein. Isothermal calorimetry shows that at 25 degrees C 3'GMP binds to a single site in barnase with a ΔC_p of $-250 \pm 50 \text{ J/(K.mol)}$. Both free barnase and ligand-bound barnase undergo a highly reversible, two-state thermal unfolding process under our experimental conditions. ΔG and ΔC_p unfolding values are similar to others found for globular

proteins, whereas ΔH and ΔS unfolding values are unusually high at the denaturation temperature of barnase. We have also found unexpectedly that the thermodynamic unfolding parameters of barnase fit neither the trend of values described in the literature for the correlation between ΔC_p and ΔH nor the limiting specific enthalpy value in the correlation between ΔH and T_m for globular proteins. These discrepancies might be related to particular features of the folded and/or unfolded states of the protein.

Matulis D., Kranz J. K., Salemme F. R., and Todd M. J. (2005) Thermodynamic stability of carbonic anhydrase: measurements of binding affinity and stoichiometry using ThermoFluor. *Biochemistry* **44**, 5258-5266.

Abstract: ThermoFluor (a miniaturized high-throughput protein stability assay) was used to analyze the linkage between protein thermal stability and ligand binding. Equilibrium binding ligands increase protein thermal stability by an amount proportional to the concentration and affinity of the ligand. Binding constants ($K(b)$) were measured by examining the systematic effect of ligand concentration on protein stability. The precise ligand effects depend on the thermodynamics of protein stability: in particular, the unfolding enthalpy. An extension of current theoretical treatments was developed for tight binding inhibitors, where ligand effect on $T(m)$ can also reveal binding stoichiometry. A thermodynamic analysis of carbonic anhydrase by differential scanning calorimetry (DSC) enabled a dissection of the Gibbs free energy of stability into enthalpic and entropic components. Under certain conditions, thermal stability increased by over 30 degrees C; the heat capacity of protein unfolding was estimated from the dependence of calorimetric enthalpy on $T(m)$. The binding affinity of six sulfonamide inhibitors to two isozymes (human type 1 and bovine type 2) was analyzed by both ThermoFluor and isothermal titration calorimetry (ITC), resulting in a good correlation in the rank ordering of ligand affinity. This combined investigation by ThermoFluor, ITC, and DSC provides a detailed picture of the linkage between ligand binding and protein stability. The systematic effect of ligands on stability is shown to be a general tool to measure affinity.

Meraz-Cruz N., Ortega A., Estrada-Gutierrez G., Flores A., Espejel A., Hernandez-Guerrero C., and Vadillo-Ortega F. (2006) Identification of a calcium-dependent matrix metalloproteinase complex in rat chorioallantoid membranes during labour. *Mol Hum Reprod* **12**, 633-641.

Abstract: The induction of the expression of matrix metalloproteinases (MMPs) and their extracellular activation are key processes in connective tissue degradation in the chorioallantoid membrane during rat labour. However, the regulatory mechanisms remain largely unknown. Here, we report the identification of a calcium-dependent high molecular weight complex composed of MMP-9, MMP-3, MMP-2, tissue inhibitor of metalloproteinase 1 (TIMP-1) and TIMP-2, identified by zymography and western blotting. Molecular sieve chromatography confirmed the presence of a complex of MMPs and TIMPs with an exclusion volume >670 kDa. Differential scanning calorimetry of the complex confirmed the existence of a macromolecular complex that unfolds with a broad transition; it is denatured over a wide range of temperatures and has a $T(m)$ of 72 degrees C in the presence of $Ca(2+)$. When denatured in the absence of $Ca(2+)$, there were at least eight transitions with $T(m)$ s that corresponded to pro-MMP-9, MMP-9, pro-MMP-3, MMP-3, pro-MMP-2, MMP-2, TIMP-1 and TIMP-2. Co-localization of the same molecular components was demonstrated by confocal microscopy using cell-depleted chorioallantoid membranes. The assembly and disassembly of the complex can be reproduced at physiological concentrations of $Ca(2+)$. This complex provides a potential mechanism for the enzymatic regulation of MMPs, which may participate in connective tissue degradation leading to the rupture of the fetal membranes during labour.

Mihalyi E. (2004) Review of some unusual effects of calcium binding to fibrinogen. *Biophys Chem* **112**, 131-140.

Abstract: Calcium binding curves of human and bovine fibrinogen were obtained by using a calcium sensitive electrode. The two were identical and showed 2 high, 2-3 medium and more than 15 low affinity sites. Differential scanning calorimetry at neutral pH demonstrated the presence of the D and E domains of fibrinogen; however, at pH 3.5 the D-domain was split into two. The presence of the subdomains was demonstrated also by digestion by pepsin at this pH. Combination of digestion of fibrinogen and of its fragments with different enzymes and temperatures identified up to 12 subdomains in the original molecule. Clotting of fibrinogen by thrombin at pH 7.0 was investigated also by differential scanning calorimetry. In the absence of Ca^{2+} clotting elicited a 40% increase in the enthalpy of thermal denaturation of the D domain of fibrinogen, but the position of the peak increased only by 0.4 degrees C. However, with clotting in the presence of $10(-3)$ M calcium the former increased by 70-75% and the latter by 11.0 degrees

C, while these parameters of the E-domain remained unchanged. Changes of bound calcium during clotting were also measured with the calcium sensitive electrode. These had to be corrected, because the drop in free calcium was partly compensated by release of some calcium that was already bound to fibrinogen. Log of the half time of calcium uptake plotted against log thrombin concentration indicated a first order process with respect to thrombin concentration, moreover, the rate determined corresponded to that of the conformation change measured by calorimetry. The calcium uptake was correlated with release of the fibrinopeptides. Release of fibrinopeptide B follows parallel to binding of calcium and that of fibrinopeptide A is about fourfold faster. Polymerization and formation of thick bundles of fibrin is connected with release of fibrinopeptide A. Clotting with Ancrod, an enzyme that releases only fibrinopeptide A, showed only minimal binding of calcium. The polymerization inhibiting tetrapeptide Gly-Pro-Arg-Pro also depressed binding of calcium. These data suggest that a calcium-binding site must be in the proximity of the site of release of fibrinopeptide B and of a polymerization site.

Mizuno K., Whittaker M. M., Bachinger H. P., and Whittaker J. W. (2004) Calorimetric studies on the tight binding metal interactions of *Escherichia coli* manganese superoxide dismutase. *J Biol Chem* **279**, 27339-27344.

Abstract: *Escherichia coli* apomanganese superoxide dismutase, prepared by removing the native metal ion under denaturing conditions, exhibits thermally triggered metal uptake behavior previously observed for thermophilic and hyperthermophilic superoxide dismutases but over a lower temperature range. Differential scanning calorimetry of apomanganese superoxide dismutase and metalated superoxide dismutase unfolding transitions has provided quantitative estimates of the metal binding affinities for manganese superoxide dismutase. The binding constant for Mn^{II} ($K_{MnII} = 3.2 \times 10^8 M^{-1}$) is surprisingly low in light of the essentially irreversible metal binding characteristic of this family of proteins and indicates that metal binding and release processes are dominated by kinetic, rather than thermodynamic, constraints. The kinetic stability of the metalloprotein complex can be traced to stabilization by elements of the protein that are independent of the presence or absence of the metal ion reflected in the thermally triggered metalation characteristic of these proteins. Binding constants for Mn^{III} , Fe^{II} , and Fe^{III} complexes were estimated using quasireversible values for the unfolding enthalpy and ΔC_p for apo-Mn superoxide dismutase and the observed T_m values for unfolding the metalated species in the absence of denaturants. For manganese and iron complexes, an oxidation state-dependent binding affinity reflects the protein perturbation of the metal redox potential.

Moll D., Prinz A., Gesellchen F., Drewianka S., Zimmermann B., and Herberg F. W. (2006) Biomolecular interaction analysis in functional proteomics. *J Neural Transm* **113**, 1015-1032.

Abstract: To understand the function of highly complex eukaryotic tissues like the human brain, in depth knowledge about cellular protein networks is required. Biomolecular interaction analysis (BIA), as a part of functional proteomics, aims to quantify interaction patterns within a protein network in detail. We used the cAMP dependent protein kinase (PKA) as a model system for the binding analysis between small natural ligands, cAMP and cAMP analogues, with their physiological interaction partner, the regulatory subunit of PKA. BIA comprises a variety of methods based on physics, biochemistry and molecular biology. Here we compared side by side real time SPR (surface plasmon resonance, Biacore), a bead based assay (AlphaScreen), a fluorescence based method (Fluorescence polarisation) and ITC (isothermal titration calorimetry). These in vitro methods were complemented by an in cell reporter assay, BRET(2) (bioluminescence resonance energy transfer), allowing to test the effects of cAMP analogues in living cells.

Nerli B., Farruggia B., and Pico G. (1996) A comparative study of the binding characteristics of ceftriaxone, cefoperazone and cefsulodin to human serum albumin. *Biochem Mol Biol Int* **40**, 823-831.

Abstract: The binding to human serum albumin of three cephalosporins of pharmacological interest: cefoperazone, ceftriaxone and cefsulodin was studied by ultrafiltration and differential scanning calorimetry methods. The identification of the binding sites in albumin was also performed using probes for the so-called sites I, II, bilirubin and fatty acids binding sites. Albumin showed two types of binding sites for cefoperazone and ceftriaxone, while for cefsulodin it showed a single type of binding site. The affinity values were: $5.6 \cdot 10^4 M^{-1}$ and $3.1 \cdot 10^4 M^{-1}$ for cefoperazone and ceftriaxone respectively, while cefsulodin showed low affinity ($3.8 \cdot 10^2 M^{-1}$). It was found that only cefoperazone interacted in a slight way with site I on serum albumin, while site II and the bilirubin binding site have capacity of binding the three cephalosporins assayed. Ceftriaxone and cefoperazone showed capacity to bind to the fatty acids binding

site on albumin. These cephalosporins increased the thermal stability of the protein, suggesting that these ligands are favouring the compact structure of the native form of the protein more than the unfolded form.

Niesen F. H., Berglund H. and Vedadi M. (2007) The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat. Protoc.* **2**, 2212-2221.

Abstract: Differential scanning fluorimetry (DSF) is a rapid and inexpensive screening method to identify low-molecular-weight ligands that bind and stabilize purified proteins. The temperature at which a protein unfolds is measured by an increase in the fluorescence of a dye with affinity for hydrophobic parts of the protein, which are exposed as the protein unfolds. A simple fitting procedure allows quick calculation of the transition midpoint; the difference in the temperature of this midpoint in the presence and absence of ligand is related to the binding affinity of the small molecule, which can be a low-molecular-weight compound, a peptide or a nucleic acid. DSF is best performed using a conventional real-time PCR instrument. Ligand solutions from a storage plate are added to a solution of protein and dye, distributed into the wells of the PCR plate and fluorescence intensity measured as the temperature is raised gradually. Results can be obtained in a single day

Nikolaeva O. P., Dedova I. V., Khvorova I. S., and Levitsky D. I. (1994) Interaction of F-actin with phosphate analogues studied by differential scanning calorimetry. *FEBS Lett* **351**, 15-18.

Abstract: The thermal unfolding of F-actin and the changes induced in it by the binding of phosphate analogues were studied by differential scanning calorimetry. It is shown that the conformation of actin is drastically altered by interaction with beryllium fluoride or aluminium fluoride, while the effects of vanadate and phosphate are negligible. The effect of beryllium fluoride on the F-actin structure, as reflected in a significant increase of the actin thermal stability, is much more pronounced in the presence of Mg²⁺ than in the case of F-actin polymerized by KCl or LiCl in the absence of Mg²⁺. It is concluded that differential scanning calorimetry is a very convenient method for probing the conformational changes in F-actin caused by the interaction with phosphate analogues.

Nikolaeva O. P., Orlov V. N., Dedova I. V., Drachev V. A., and Levitsky D. I. (1996) Interaction of myosin subfragment 1 with F-actin studied by differential scanning calorimetry. *Biochem Mol Biol Int* **40**, 653-661.

Abstract: The thermal unfolding of the myosin subfragment 1 (S1) and of filamentous actin (F-actin) in their strong complex obtained in the presence of ADP was studied by differential scanning calorimetry (DSC). It is shown that in the acto-S1 complexes S1 and F-actin melt separately, and thermal transitions of each protein can be easily followed. Interaction of S1 with F-actin significantly increases S1 thermal stability and also affects the thermal stability of F-actin. Although S1 unfolds at much lower temperature than F-actin, the molecules of S1 remain bound to F-actin even after full denaturation. Under these conditions S1 may induce cross-linking between actin filaments. It is concluded that DSC studies on the acto-S1 complexes offer a new and promising approach to investigate the structural changes which occur in the myosin head and in F-actin due to their interaction.

Orioni B., Roversi M., La Mesa C., Asaro F., Pellizer G., and D'Errico G. (2006) Polymorphic behavior in protein-surfactant mixtures: the water-bovine serum albumin-sodium taurodeoxycholate system. *J Phys Chem B Condens Matter Mater Surf Interfaces Biophys* **110**, 12129-12140.

Abstract: Mixtures containing water, bovine serum albumin (BSA), and sodium taurodeoxycholate (NaTDC), a component of the bile in mammals, have been investigated in a wide range of composition and pH. Depending on the concentration of both solutes and the pH, solutions, precipitates, and gels are formed. Under spontaneous pH conditions, the transport properties in dilute solutions indicate the occurrence of significant interactions between BSA and the surfactant. Conversely, acidic media favor the formation of nonsoluble protein-surfactant complexes, with subsequent precipitation. The nucleation kinetics of the protein-surfactant complexes in solid form and the related precipitation processes can be slow or fast, depending on the overall solute content and the mole ratio. At high concentrations, a gel, extending on both sides of the charge neutralization line, and two-phase regions are observed. Gels shrink in open air and swell in the presence of excess water. Depending on concentration and temperature, the gels transform from an essentially liquidlike behavior to that peculiar to true gels (when $G' > \text{or} = G''$). The thermal gelation threshold, the temperature above which $G' > \text{or} = G''$, depends on BSA and NaTDC content and is concomitant to moderate heat effects, inferred by differential scanning calorimetry (DSC). The above data also indicate that the protein thermal denaturation in the gel is shifted to higher temperatures compared to

water. Such a stabilizing effect is presumably related to the occurrence of both electrostatic and hydrophobic interactions with NaTDC. Water self-diffusion in the gels is slightly slower than that in the bulk and poorly sensitive to composition: it is about 65% the value of neat H₂O in a wide concentration range, irrespective of the BSA, or NaTDC, concentration. A peculiar behavior is also observed in ²³Na longitudinal and transverse relaxation rates. The T₁ and T₂ values, measured at 105.75 MHz on BSA-NaTDC gels, indicate that the motions determining the NMR relaxation of the sodium ions in the hydration layer of the protein-surfactant aggregates are not slow, having frequencies comparable with the Larmor one. The above properties, especially the rheological and the spectroscopic ones, are important for understanding the behavior of gels based on protein-surfactant mixtures.

Panyukov Y. V., Nemykh M. A., Dobrov E. N. and Drachev V. A. (2008) Surfactant-induced amorphous aggregation of tobacco mosaic virus coat protein: a physical methods approach. *Macromol. Biosci.* **8**, 199-209.

Abstract: The interactions of non-ionic surfactant Triton X-100 and the coat protein of tobacco mosaic virus, which is an established model for both ordered and non-ordered protein aggregation, were studied using turbidimetry, differential scanning calorimetry, isothermal titration calorimetry, and dynamic light scattering. It was found that at the critical aggregation concentration (equal to critical micelle concentration) of 138×10^{-6} M, Triton X-100 induces partial denaturation of tobacco mosaic virus coat protein molecules followed by protein amorphous aggregation. Protein aggregation has profound ionic strength dependence and proceeds due to hydrophobic sticking of surfactant-protein complexes (start aggregates) with initial radii of 46 nm. It has been suggested that the anionic surfactant sodium dodecyl sulfate forms mixed micelles with Triton X-100 and therefore reverses protein amorphous aggregation with release of protein molecules from the amorphous aggregates. A stoichiometric ratio of 5 was found for Triton X-100-sodium dodecyl sulfate interactions

Piszczek G., D'Auria S., Staiano M., Rossi M., and Ginsburg A. (2004) Conformational stability and domain coupling in D-glucose/D-galactose-binding protein from *Escherichia coli*. *Biochem J* **381**, 97-103.

Abstract: The monomeric D-glucose/D-galactose-binding protein (GGBP) from *Escherichia coli* (M_r 33000) is a periplasmic protein that serves as a high-affinity receptor for the active transport and chemotaxis towards both sugars. The effect of D-glucose binding on the thermal unfolding of the GGBP protein at pH 7.0 has been measured by differential scanning calorimetry (DSC), far-UV CD and intrinsic tryptophanyl residue fluorescence (Trp fluorescence). All three techniques reveal reversible, thermal transitions and a midpoint temperature (T_m) increase from 50 to 63 degrees C produced by 10 mM D-glucose. Both in the absence and presence of D-glucose a single asymmetric endotherm for GGBP is observed in DSC, although each endotherm consists of two transitions about 4 degrees C apart in T_m values. In the absence of D-glucose, the protein unfolding is best described by two non-ideal transitions, suggesting the presence of unfolding intermediates. In the presence of D-glucose protein, unfolding is more co-operative than in the absence of the ligand, and the experimental data are best fitted to a model that assumes two ideal (two-state) sequential transitions. Thus D-glucose binding changes the character of the GGBP protein folding/unfolding by linking the two domains such that protein unfolding becomes a cooperative, two two-state process. A K_A' value of $5.6 \times 10^6 \text{ M}^{-1}$ at 63 degrees C for D-glucose binding is estimated from DSC results. The domain with the lower stability in DSC measurements has been identified as the C-terminal domain of GGBP from thermally induced Trp fluorescence changes.

Plotnikov, V., Rochalski, A., Brandts, M., Brandts, J. F., Williston, S., Frasca, V., and Lin, L.N. (2002) An Autosampling Differential Scanning Calorimeter Instrument for Studying Molecular Interactions. *ASSAY and Drug Development Technologies* **1**, 83-90.

Abstract: A new ultrasensitive differential scanning calorimeter (DSC) instrument is described, which utilizes autosampling for continuous operation. High scanning rates to 250 deg/h with rapid cooling and equilibration between scans facilitates higher sample throughput up to 50 samples during each 24 h of unattended operation. The instrument is suited for those pharmaceutical applications where higher throughput is important, such as screening drug candidates for binding constant or screening solution conditions for stability of liquid protein formulations. Results are presented on the binding of five different anionic inhibitors to ribonuclease A, which included cytidine 29-monophosphate (29CMP), 39CMP, uridine 39-monophosphate, pyrophosphate, and phosphate. Binding constants K_B (or dissociation constants K_d) are obtained from the shift in the transition

temperature T_M for ribonuclease thermal unfolding in the presence of ligand relative to the transition temperature in the absence of ligand. Measured binding constants ranged from 155 M^{-1} ($K_d = 6.45$ mM) for the weak-binding phosphate anion to 13,100 M^{-1} ($K_d = 76.3$ mM) for the strongest binding ligand, 29CMP. The DSC method for measuring binding constants can also be extended to ultratight interactions involving either ligand–protein or protein–protein binding.

Quesada-Soriano I., Musso-Buendia J. A., Tellez-Sanz R., Ruiz-Perez L. M., Baron C., Gonzalez-Pacanoska D. and Garcia-Fuentes L. (2007) Plasmodium falciparum dUTPase: studies on protein stability and binding of deoxyuridine derivatives. *Biochim Biophys Acta* **1774**, 936-945.

Abstract: Deoxyuridine triphosphate nucleotidohydrolase (dUTPase), a ubiquitous enzyme preventing a deleterious incorporation of uracil into DNA, has been thought of as a novel target for anticancer and antiviral drug design. The interaction of Plasmodium falciparum dUTPase (PfdUTPase) with deoxyuridine derivatives (dU, dUMP, dUDP and dUpNHpp) has been studied thermodynamically by both isothermal titration and differential scanning calorimetry. ITC shows no cooperativity for the binding of these derivatives. Dependencies in the binding thermodynamic parameters (enthalpy, entropy and Gibbs energy changes) with the number of phosphate groups in the nucleotide are obtained, and from the heat capacity changes no significant conformational changes upon binding are inferred. DSC shows PfdUTPase trimer is very stable but denatures irreversibly, with a more complex denaturation profile than other homologous trimeric dUTPases. The presence of magnesium ions does not influence the denaturation profile, while the presence of deoxyuridine derivatives increases the stability. The increase depends upon nucleotide concentration and type, with dUDP having the greater effect.

Rahuel-Clermont S., Arutyunov D., Marchal S., Orlov V., Muronetz V., and Branlant G. (2005) Thermal destabilization of non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from Streptococcus mutans upon phosphate binding in the active site. *J Biol Chem* **280**, 18590-18597.

Abstract: Catalysis by the NADP-dependent non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) from Streptococcus mutans, a member of the aldehyde dehydrogenase (ALDH) family, relies on a local conformational reorganization of the active site. This rearrangement is promoted by the binding of NADP and is strongly kinetically favored by the formation of the ternary complex enzyme.NADP.substrate. Adiabatic differential scanning calorimetry was used to investigate the effect of ligands on the irreversible thermal denaturation of GAPN. We showed that phosphate binds to GAPN, resulting in the formation of a GAPN.phosphate binary complex characterized by a strongly decreased thermal stability, with a difference of at least 15 degrees C between the maximum temperatures of the thermal transition peaks. The kinetics of phosphate association and dissociation are slow, allowing both free and GAPN.phosphate complexes to be observed by differential scanning calorimetry and to be separated by native polyacrylamide electrophoresis run in phosphate buffer. Analysis of a set of mutants of GAPN strongly suggests that phosphate is bound to the substrate C-3 subsite. In addition, the substrate analog glycerol-3-phosphate has similar effects as does phosphate on the thermal behavior of GAPN. Based on the current knowledge on the catalytic mechanism of GAPN and other ALDHs, we propose that ligand-induced thermal destabilization is a mechanism that provides to ALDHs the required flexibility for an efficient catalysis.

Repo S., Paldanius T. A., Hytonen V. P., Nyholm T. K., Halling K. K., Huuskonen J., Pentikainen O. T., Rissanen K., Slotte J. P., Airene T. T., Salminen T. A., Kulomaa M. S., and Johnson M. S. (2006) Binding properties of HABA-type azo derivatives to avidin and avidin-related protein 4. *Chem Biol* **13**, 1029-1039.

Abstract: The chicken genome encodes several biotin-binding proteins, including avidin and avidin-related protein 4 (AVR4). In addition to D-biotin, avidin binds an azo dye compound, 4-hydroxyazobenzene-2-carboxylic acid (HABA), but the HABA-binding properties of AVR4 are not yet known. Differential scanning calorimetry, UV/visible spectroscopy, and molecular modeling were used to analyze the binding of 15 azo molecules to avidin and AVR4. Significant differences are seen in azo compound preferences for the two proteins, emphasizing the importance of the loop between strands beta3 and beta4 for azo ligand recognition; information on these loops is provided by the high-resolution (1.5 Å) X-ray structure for avidin reported here. These results may be valuable in designing improved tools for avidin-based life science and nanobiotechnology applications.

Rippin T. M., Bykov V. J., Freund S. M., Selivanova G., Wiman K. G., and Fersht A. R. (2002) Characterization of the p53-rescue drug CP-31398 in vitro and in living cells. *Oncogene* **21**, 2119-2129.
Abstract: The Pfizer compound CP-31398 has been reported to stabilize the core domain of the tumour suppressor p53 in vitro and be an effective anti-cancer drug by virtue of rescuing destabilized mutants of p53. We did not detect any interaction between the p53 core domain and CP-31398 in vitro by a wide range of quantitative biophysical techniques over a wide range of conditions. CP-31398 did not stabilize p53 in our experiments. However, we found that CP-31398 intercalated with DNA and also altered and destabilized the DNA-p53 core domain complex. We analysed by NMR TROSY the interaction of the domain with a DNA oligomer and identified the changes in the complex on the binding of CP-31398. CP-31398 also decreased sequence-specific DNA binding of wild-type p53 and His-273 mutant p53. CP-31398 had a non-specific toxic effect independent of mutant p53 expression in several cell lines carrying Tet-regulated mutant p53. CP-31398 caused a small increase in MDM-2 expression and a more pronounced p53-independent increase in Bax expression. CP-31398 did, however, induce the PAb1620 epitope (characteristic of native p53) in cells expressing His-175 mutant p53. This was prevented by cycloheximide, suggesting that any stabilizing action of CP-31398 would have to be on newly synthesized p53. One of the unstable mutants that was reported to have been rescued by CP-31398, R249S, does not bind DNA when folded at lower temperatures.

Rochu D., Clery-Barraud C., Renault F., Chevalier A., Bon C., and Masson P. (2006) Capillary electrophoresis versus differential scanning calorimetry for the analysis of free enzyme versus enzyme-ligand complexes: in the search of the ligand-free status of cholinesterases. *Electrophoresis* **27**, 442-451.
Abstract: Cholinesterases (ChEs) are highly efficient biocatalysts whose active site is buried in a deep, narrow gorge. The talent of CE to discover inhibitors in the gorge of highly purified preparations has fairly altered the meaning of a ChE ligand-free status. To attempt at a description of this one, we investigated the stability of Bungarus fasciatus acetylcholinesterase (AChE), alone or complexed with different inhibitors. Determination of mid-transition temperature for thermal denaturation, using differential scanning calorimetry (DSC) and CE, provided conflicting results. Discrepancies strongly question the reality of a ligand-free AChE state. DSC allowed estimation of the stability of AChE-ligands complexes, and to rank the stabilizing effect of different inhibitors. CE acted as a detector of hidden ligands, provided that they were charged, reversibly bound, and thus dissociable upon action of electric fields. Then, CE allowed quantification of the stability of ligand-free AChE. CE and DSC providing each fractional and nonredundant information, cautious attention must be paid for actual estimation of the conformational stability of ChEs. Because inhibitors used in purification of ChEs by affinity chromatography are charged, CE remains a leading method to estimate enzyme stability and detect the presence of bound hidden ligands.

Rondeau J. M., Bitsch F., Bourcier E., Geiser M., Hemmig R., Kroemer M., Lehmann S., Ramage P., Rieffel S., Strauss A., Green J. R., and Jahnke W. (2006) Structural basis for the exceptional in vivo efficacy of bisphosphonate drugs. *ChemMedChem* **1**, 267-273.
Abstract: To understand the structural basis for bisphosphonate therapy of bone diseases, we solved the crystal structures of human farnesyl pyrophosphate synthase (FPPS) in its unliganded state, in complex with the nitrogen-containing bisphosphonate (N-BP) drugs zoledronate, pamidronate, alendronate, and ibandronate, and in the ternary complex with zoledronate and the substrate isopentenyl pyrophosphate (IPP). By revealing three structural snapshots of the enzyme catalytic cycle, each associated with a distinct conformational state, and details about the interactions with N-BPs, these structures provide a novel understanding of the mechanism of FPPS catalysis and inhibition. In particular, the accumulating substrate, IPP, was found to bind to and stabilize the FPPS-N-BP complexes rather than to compete with and displace the N-BP inhibitor. Stabilization of the FPPS-N-BP complex through IPP binding is supported by differential scanning calorimetry analyses of a set of representative N-BPs. Among other factors such as high binding affinity for bone mineral, this particular mode of FPPS inhibition contributes to the exceptional in vivo efficacy of N-BP drugs. Moreover, our data form the basis for structure-guided design of optimized N-BPs with improved pharmacological properties.

Rosengarth A., Rosgen J., Hinz H. J., and Gerke V. (2001) Folding energetics of ligand binding proteins II. Cooperative binding of Ca²⁺ to annexin I. *J Mol Biol* **306**, 825-835.
Abstract: The calcium binding properties of annexin I as observed by thermodynamic DSC studies have been compared to the structural information obtained from X-ray investigation. The calorimetric

experiment permitted to evaluate both the reaction scheme - including binding of ligand and conformational changes - and the energetics of each reaction step. According to published X-ray data Annexin I has six calcium binding sites, three medium-affinity type II and three low-affinity type III sites. The present study shows that at 37 degrees C annexin I binds in a Hill type fashion simultaneously two calcium ions in a first step with medium affinity at a concentration of 0.6 mM and another three Ca(2+) ions again cooperatively at 30 mM with low affinity. Therefore it can be concluded that only two medium-affinity type II binding sites are available. The third site, that should be accessible in principle appears to be masked presumably due to the presence of the N terminus. In view of the large calcium concentration needed for saturation of the binding sites, annexin I may be expected to be Ca(2+) free in vivo unless other processes such as membrane interaction occur simultaneously. This assumption is consistent with the finding, that the affinity of annexins to calcium is usually markedly increased by the presence of lipids.

Rosgen J. and Hinz H. J. (2001) Folding energetics of ligand binding proteins. I. Theoretical model. *J Mol Biol* **306**, 809-824.

Abstract: Heat capacity curves as obtained from differential scanning calorimetry are an outstanding source for molecular information on protein folding and ligand-binding energetics. However, deconvolution of Cp data of proteins in the presence of ligands can be compromised by indeterminacies concerning the correct choice of the statistical thermodynamic ensemble. By convention, the assumption of constant free ligand concentration has been used to derive formulae for the enthalpy. Unless the ligand occurs at large excess, this assumption is incorrect. Still the relevant ensemble is the grand canonical ensemble. We derive formulae for both constraints, constancy of total or free ligand concentration and illustrate the equations by application to the typical equilibrium $Nx \rightleftharpoons N + x \rightleftharpoons D + x$. It is demonstrated that as long as the thermodynamic properties of the ligand can be completely corrected for by performing a reference measurement, the grand canonical approach provides the proper and mathematically significantly simpler choice. We demonstrate on the two cases of sequential or independent ligand-binding the fact, that similar binding mechanisms result in different and distinguishable heat capacity equations. Finally, we propose adequate strategies for DSC experiments as well as for obtaining first estimates of the characteristic thermodynamic parameters, which can be used as starting values in a global fit of DSC data.

Rosgen J. and Hinz H. J. (2002) The heat capacity paradox of ligand binding proteins: reconciling the microscopic and macroscopic world. *Biophys Chem* **96**, 109-116.

Abstract: Differential scanning microcalorimetry (DSC) is a superb method for the analysis of protein energetics. However, the relative simplicity of application has led astray many to assume that a proper analysis of the data was possible without a sound knowledge of the underlying statistical thermodynamic principles. In this study, the question is addressed of how to calculate properly the heat capacity signal of a protein in the presence of high affinity ligands. It is shown that the signal corresponds neither to grand canonic nor to canonic heat capacity. Statistical thermodynamic model calculations result only in the observed macroscopic heat capacity signal, if the protein in the calorimetric cell is assumed to form a grand canonic ensemble (T, p, μ controlled) which is, however, heated under constraints typical for a canonic ensemble (T, p, N controlled). As a consequence, the microscopic statistical thermodynamic heat capacity must be carefully distinguished from the macroscopically observable thermodynamic heat capacity in those cases where proteins unfold in the presence of high affinity ligands.

Rosgen J. and Hinz H. J. (2003) Phase diagrams: a graphical representation of linkage relations. *J Mol Biol* **328**, 255-271.

Abstract: It is shown here that phase diagrams of ligand-binding biological macromolecules provide a powerful tool for the analysis of reaction mechanisms. The present study provides simple rules for the construction and interpretation of such phase diagrams. We give examples for the derivation of reaction schemes for macromolecules that can bind two different kinds of ligands. By sampling one dimension of a phase diagram it is possible to reconstruct the second dimension, including the correct stoichiometry, positive and negative linkage between the ligands and equilibrium binding constants for the complete series of reactions. The discussion is generalised to temperature and pressure-dependent phase diagrams. To exemplify the new diagram method we analyse the pH-dependent binding of trans-beta-indole acrylic acid to apo-Trp repressor, the pH-dependent thermal denaturation of alpha-chymotrypsinogen A, calcium binding and denaturation of annexin I, high affinity zinc binding to a metallo-beta-lactamase and high-pressure and temperature denaturation of RNase A and staphylococcal nuclease.

Rosso S. B., Gonzalez M., Bagatolli L. A., Duffard R. O., and Fidelio G. D. (1998) Evidence of a strong interaction of 2,4-dichlorophenoxyacetic acid herbicide with human serum albumin. *Life Sci* **63**, 2343-2351.

Abstract: The interaction of 2,4-dichlorophenoxyacetic acid herbicide (2,4-D) with human serum albumin (HSA) was studied using fluorescence and differential scanning calorimetry (DSC). Fluorescence displacement of 1-anilino-8-naphthalenesulfonate (ANS) bound to HSA was used to evaluate the binding affinity of 2,4-D to HSA. The binding is associated to a high affinity site of HSA located in the IIIA subdomain. The association constant (K_{ass}) of the herbicide was about $5 \mu\text{M}^{-1}$, several times higher than the affinity found for pharmaceutical compounds. This relatively strong interaction with HSA was evidenced by the increase in HSA protein thermostability induced as consequence of herbicide interaction. 2,4-D induces an increase in the midpoint of thermal denaturation temperature from 60.1 degrees C in herbicide free solution to 75.6 degrees C in full ligand saturating condition. The calorimetric enthalpy and the excess heat capacity also increased upon 2,4-D binding. To investigate the possibility of other/s system/s of 2,4-D transport in blood, besides of HSA, the interaction of the herbicide with lipid monolayers was explored. No interaction was detected with any of the lipids tested. The overall results provided evidence that high affinity 2,4-D-HSA complex exhibits enhanced thermal stability and that HSA is the unique transport system for 2,4-D in blood.

Rozhkov S. P., Goryunov A. S., Sukhanova G. A., Borisova A. G., Rozhkova N. N., and Andrievsky G. V. (2003) Protein interaction with hydrated C60 fullerene in aqueous solutions. *Biochem Biophys Res Commun* **303**, 562-566.

Abstract: Physicochemical effects of hydrated C(60) fullerenes (HyFn) on serum albumin molecules were studied using ESR spin labeling and differential scanning microcalorimetry. Molecular-colloidal solution of hydrated C(60) fullerenes and their small spherical fractal clusters in water (C(60)FWS), was shown to stabilize protein hydration, and decrease specific surface energy in water-protein matrix in salt solutions. The mechanism of HyFn interaction with protein is discussed in terms of HyFn induced formation of protein clusters and phase transition of hydration water.

Sagar S. L. and Domach M. M. (1995) Using differential scanning calorimetry to elucidate metal-protein binding sites in alpha- and gamma-chymotrypsin. *Bioseparation* **5**, 289-294.

Abstract: Crystalline alpha-chymotrypsin preparations are contaminated by the post translational variant, gamma-chymotrypsin. The contaminant can account for 5-50 weight percent of the preparation based on thermal analysis. Such contamination can be problematic because this serine protease has both commercial and deactivation model system utility, and the presence of the contaminant may not be detectable by activity assays. Prior work has shown that simple pH gradient elution can separate the two chymotrypsins when loaded to a Cu(2+)-IMAC column; gamma-chymotrypsin eluted first indicating that its interaction with immobilized Cu²⁺ is weaker. The molecular features that endow these serine proteases with metal affinity has been investigated further by performing differential scanning calorimetry (DSC) studies in the presence and absence of Cu²⁺, and at different pH values. The dependence of thermostability on pH for fixed metal concentration reveals an interplay between stabilizing and destabilizing metal binding events. The results are consistent with Cu(2+)-chymotrypsin interaction occurring, in part, through binding to a glutamate- or aspartate-containing chelation site. The strength of this site may differ in the two chymotrypsins.

Sarver R. W., Rogers J. M., and Epps D. E. (2002) Determination of ligand-MurB interactions by isothermal denaturation: application as a secondary assay to complement high throughput screening. *J Biomol Screen* **7**, 21-28.

Abstract: We used a temperature-jump isothermal denaturation procedure with various methods of detection to evaluate the quality of putative inhibitors of MurB discovered by high-throughput screening. Three optical methods of detection-ultraviolet hyperchromicity of absorbance, fluorescence of bound dyes, and circular dichroism-as well as differential scanning calorimetry were used to dissect the effects of two chemical compounds and a natural substrate on the enzyme. The kinetics of the denaturation process and binding of the compounds detected by quenching of flavin fluorescence were used to quantitate the dose dependencies of the ligand effects. We found that the first step in the denaturation of MurB is the rapid loss of flavin from the active site and that the two chemical inhibitors appeared to destabilize the interaction of the cofactor with the enzyme but stabilize the global unfolding. The kinetics of the denaturation process as

well as the loss of flavin fluorescence on binding established that both compounds had nanomolar affinities for the enzyme. We showed that coupling of the various detection methods with isothermal denaturation yields a powerful regimen to provide analytical data for assessing inhibitor specificity for a protein target.

Sasso S., Protasevich I., Gilli R., Makarov A., and Briand C. (1995) Thermal denaturation of bacterial and bovine dihydrofolate reductases and their complexes with NADPH, trimethoprim and methotrexate. *J Biomol Struct Dyn* **12**, 1023-1032.

Abstract: Scanning microcalorimetry was used for the study of thermal denaturation of E.coli and bovine liver dihydrofolate reductases (cDHFR and bDHFR, respectively) and their complexes with NADPH, trimethoprim (TMP) and methotrexate (MTX) at pH 6.8. It was shown that the denaturation temperature of bDHFR is 7.2 degrees C less than that of cDHFR and that ionic strength is equally important for the thermostability and cooperativity of the denaturation process of the two proteins. Binding of antifolate compounds significantly stabilizes DHFR against heat denaturation. The stabilizing effect and the transition cooperativity depend on the nature of the inhibitor, the presence of NADPH and the origin of the enzyme. The dependence of calorimetric denaturation enthalpy (calculated per gram of protein) on denaturation temperature for DHFRs, their complexes with NADPH and binary/ternary complexes with TMP/MTX fits to the same straight line with the slope of 0.66 J/Kg. This relatively high value indicates an essential role of hydrophobic contacts in the stabilization of DHFR structure. The change of denaturation temperatures in binary complexes with MTX/TMP (in comparison with the free enzymes) is as much as 14.2 degrees C/8.5 degrees C and 13.3 degrees C/3.2 degrees C for cDHFR and bDHFR, respectively. The same change in ternary complexes with MTX/TMP is much more pronounced and equals to 21.9 degrees C/16.8 degrees C and 29.0 degrees C/16.4 degrees C. The vast difference of binary and ternary complexes thermostability demonstrates the important role of cofactor in the stabilization of enzyme. Moving from binary to ternary systems causes a significant increase in denaturation temperatures, even when corresponding association constants do not change (cDHFR binary/ternary complexes with MTX) or increases very slightly (bDHFR binary/ternary complexes with TMP). In all other cases the increase of denaturation temperature for each protein in complex with ligands correlates with the association constant for the corresponding complex.

Sayer J. M. and Louis J. M. (2008) Interactions of different inhibitors with active-site aspartyl residues of HIV-1 protease and possible relevance to pepsin. *Proteins (publication)*.

Abstract: The importance of the active site region aspartyl residues 25 and 29 of the mature HIV-1 protease (PR) for the binding of five clinical and three experimental protease inhibitors [symmetric cyclic urea inhibitor DMP323, nonhydrolyzable substrate analog (RPB) and the generic aspartic protease inhibitor acetyl-pepstatin (Ac-PEP)] was assessed by differential scanning calorimetry. DeltaT(m) values, defined as the difference in T(m) for a given protein in the presence and absence of inhibitor, for PR with DRV, ATV, SQV, RTV, APV, DMP323, RPB, and Ac-PEP are 22.4, 20.8, 19.3, 15.6, 14.3, 14.7, 8.7, and 6.5 degrees C, respectively. Binding of APV and Ac-PEP is most sensitive to the D25N mutation, as shown by DeltaT(m) ratios [DeltaT(m)(PR)/DeltaT(m)(PR(D25N))] of 35.8 and 16.3, respectively, whereas binding of DMP323 and RPB (DeltaT(m) ratios of 1-2) is least affected. Binding of the substrate-like inhibitors RPB and Ac-PEP is nearly abolished (DeltaT(m)(PR)/DeltaT(m)(PR(D29N)) >= 44) by the D29N mutation, whereas this mutation only moderately affects binding of the smaller inhibitors (DeltaT(m) ratios of 1.4-2.2). Of the nine FDA-approved clinical HIV-1 protease inhibitors screened, APV, RTV, and DRV competitively inhibit porcine pepsin with K(i) values of 0.3, 0.6, and 2.14 muM, respectively. DSC results were consistent with this relatively weak binding of APV (DeltaT(m) 2.7 degrees C) compared with the tight binding of Ac-PEP (DeltaT(m) >= 17 degrees C). Comparison of superimposed structures of the PR/APV complex with those of PR/Ac-PEP and pepsin/pepstatin A complexes suggests a role for Asp215, Asp32, and Ser219 in pepsin, equivalent to Asp25, Asp25', and Asp29 in PR in the binding and stabilization of the pepsin/APV complex. *Proteins* 2008. Published 2008 Wiley-Liss, Inc

Sayer J. M., Liu F., Ishima R., Weber I. T. and Louis J. M. (2008) Effect of the active site D25N mutation on the structure, stability, and ligand binding of the mature HIV-1 protease. *J Biol Chem* **283**, 13459-13470.

Abstract: All aspartic proteases, including retroviral proteases, share the triplet DTG critical for the active site geometry and catalytic function. These residues interact closely in the active, dimeric structure of HIV-1 protease (PR). We have systematically assessed the effect of the D25N mutation on the structure and stability of the mature PR monomer and dimer. The D25N mutation (PR(D25N)) increases the equilibrium

dimer dissociation constant by a factor >100-fold (1.3 +/- 0.09 microm) relative to PR. In the absence of inhibitor, NMR studies reveal clear structural differences between PR and PR(D25N) in the relatively mobile P1 loop (residues 79-83) and flap regions, and differential scanning calorimetric analyses show that the mutation lowers the stabilities of both the monomer and dimer folds by 5 and 7.3 degrees C, respectively. Only minimal differences are observed in high resolution crystal structures of PR(D25N) complexed to darunavir (DRV), a potent clinical inhibitor, or a non-hydrolyzable substrate analogue, Ac-Thr-Ile-Nle-r-Nle-Gln-Arg-NH(2) (RPB), as compared with PR.DRV and PR.RPB complexes. Although complexation with RPB stabilizes both dimers, the effect on their T(m) is smaller for PR(D25N) (6.2 degrees C) than for PR (8.7 degrees C). The T(m) of PR(D25N).DRV increases by only 3 degrees C relative to free PR(D25N), as compared with a 22 degrees C increase for PR.DRV, and the mutation increases the ligand dissociation constant of PR(D25N).DRV by a factor of approximately 10(6) relative to PR.DRV. These results suggest that interactions mediated by the catalytic Asp residues make a major contribution to the tight binding of DRV to PR

Sedlak E., Antalík M., Bagel'ova J., and Fedurco M. (1997) Interaction of ferricytochrome c with polyanion Nafion. *Biochim Biophys Acta* **1319**, 258-266.

Abstract: The properties of the complex of ferricyt c with fluorosulfonated polyanion Nafion (as a representative 'hydrophobic' polyanion) have been studied by means of optical spectroscopy and differential scanning calorimetry. The addition of the polyanion to a solution of ferricyt c (pH 7.4) resulted in an expansion of the protein molecule characterized by a profound decrease in enthalpy of the thermal transition of ferricyt c. The conformational change of ferricyt c upon addition of Nafion was shown by a perturbation of the Met-80-heme iron bond and an apparent increase in the distance of Trp-59 from the heme. The conformational change in the heme region was also observed by examining the CD spectra. The conformational state of ferricyt c in a complex with Nafion was similar to that designated as state II by Hildebrandt (Hildebrandt, P. (1990) *Biochim. Biophys. Acta* 1040, 175-186) in the complex with negatively charged heteropolytungstates—a six-coordinated low-spin state with a destabilized structure of the heme crevice. The decrease in enthalpy of the thermal transition of ferricyt c, the spectral changes in absorbance and the CD spectra, together with an increase in Trp fluorescence induced by Nafion addition observed at high ionic strength, all point to the involvement of the non-coulombic interaction.

Sedlak E. and Antalík M. (1998) Coulombic and noncoulombic effect of polyanions on cytochrome c structure. *Biopolymers* **46**, 145-154.

Abstract: The properties of the complexes of ferricytochrome c with two different polyanions--poly(vinylsulfate) and poly(4-styrene-sulfonate)--with a comparable charge density but with the different size of the uncharged part of their molecules have been studied by means of optical spectroscopy, differential scanning calorimetry, and gel chromatography. Ferricytochrome c formed a complex with the former one through coulombic interactions and remained in a native-like state. The addition of the second polyanion to a solution of ferric cytochrome c at a low ionic strength, pH 7.0, resulted in profound conformational change in the hydrophobic core of protein (opening of the heme crevice with a perturbation of the methionine 80-heme iron bond and the hydrophobic core of the protein). These may be understood as an involvement of noncoulombic (hydrophobic, H-bonding) interactions of the uncharged part of the polyanion molecule. Conformational changes and the observed shift in acidic transition from low spin to high spin state of ferric cytochrome c detected in the presence of the polyanions may have biological implication in understanding the origin of conformational changes in proteins induced in the course of their interaction with membrane lipids and membrane proteins.

Sedlak E. and Antalík M. (1999) Molten globule-like state of cytochrome c induced by polyanion poly(vinylsulfate) in slightly acidic pH. *Biochim Biophys Acta* **1434**, 347-355.

Abstract: The effect of polyanion, poly(vinylsulfate), used as a model of negatively charged surface, on ferric cytochrome c (ferricyt c) structure in acidic pH has been studied by absorbance spectroscopy, circular dichroism (CD), tryptophan (Trp) fluorescence and microcalorimetry. The polyanion induced only small changes in the native structure of the protein at neutral pH, but it profoundly shifted the acid induced high spin state of the heme in the active center of cyt c to a more neutral pH region. Cooperativity of the acidic transition of ferricyt c in the presence of the polyanion was disturbed, in comparison with uncomplexed protein, as followed from different apparent pK(a) values observed in a distinct regions of the ferricyt c electronic absorbance spectrum (4.55+/-0.08 in the 620 nm band region and 5.47+/-0.15 in the Soret

region). The ferricyt c structure in the complex with the polyanion at acidic pH (below pH 5.0) has properties of a molten globule-like state. Its tertiary structure is strongly disturbed according to CD and microcalorimetry measurements; however, its secondary structure, from CD, is still native-like and ferricyt c is in a compact state as evidenced by quenched Trp fluorescence. These findings are discussed in the context of the molten globule state of proteins induced on a negatively charged membrane surface under physiological conditions.

Sedlak E., Zoldak G., Antalík M., and Sprinzl M. (2002) Thermodynamic properties of nucleotide-free EF-Tu from *Thermus thermophilus* in the presence of low-molecular weight effectors of its GTPase activity. *Biochim Biophys Acta* **1597**, 22-27.

Abstract: The thermal transition of elongation factor EF-Tu from *Thermus thermophilus* in the presence of low-molecular weight effectors was studied by differential scanning calorimetry. The effectors of GTPase activity used were the antibiotic kirromycin and the cations Li(+), Na(+), K(+) and NH₄(+) in the chloride form. The temperature of thermal denaturation and the cooperativity of the transition of nucleotide-free EF-Tu (EF-Tu(f)) in the presence of kirromycin are comparable with those of the EF-Tu x guanosine-5'-[beta,gamma-imido]triphosphate (GppNHp) form, indicating similar conformational states. Increased concentrations of Na(+) and K(+) stabilized EF-Tu(f) in a manner similar to GppNHp. NH₄(+) decreased the transition temperature of EF-Tu(f) and Li(+) decreased both the temperature and the calorimetric enthalpy of the thermal transition of EF-Tu(f). In the presence of salts, binding of kirromycin had a stabilizing effect on EF-Tu(f). Correlation between the GTPase activity and thermodynamic characteristics of EF-Tu(f) induced by kirromycin in the absence or presence of the cations is discussed.

Sedlak E., Sprinzl M., Grillenbeck N., and Antalík M. (2002) Microcalorimetric study of elongation factor Tu from *Thermus thermophilus* in nucleotide-free, GDP and GTP forms and in the presence of elongation factor Ts. *Biochim Biophys Acta* **1596**, 357-365.

Abstract: Elongation factor (EF) Tu undergoes profound nucleotide-dependent conformational changes in its functional cycle. The thermodynamic parameters of the different *Thermus thermophilus* EF-Tu forms, its domains I, II/III and III, were determined by microcalorimetry. Thermal transitions of the EF-Tu.GDP and EF-Tu.guanosine-5'-[beta,gamma-imido]triphosphate have a cooperative two-state character. Nucleotide removal affected the cooperativity of the thermal transition of EF-Tu. Microcalorimetric measurements of nucleotide-free EF-Tu and its separated domains showed that domains II/III have the main stabilizing role for the whole protein. Despite the fact that strong interactions between elongation factors Tu and Ts from *T. thermophilus* at 20 degrees C exist, the thermal transition of neither protein in the complex was significantly affected.

Shrake A., Frazier D., and Schwarz F. P. (2006) Thermal stabilization of human albumin by medium- and short-chain n-alkyl fatty acid anions. *Biopolymers* **81**, 235-248.

Abstract: A comprehensive study of the thermal stabilization of defatted human albumin monomer by n-alkyl fatty acid anions (FAAs), formate through n-decanoate, was carried out by differential scanning calorimetry (DSC). The concentration of each ligand affording maximum thermal stabilization was determined; n-nonanoate provides the greatest stabilization but is only marginally better than n-octanoate and n-decanoate. The use of reversible thermodynamics and a two-state denaturation model for albumin has been validated. Standard free energies of binding, calculated from increases in free energy of denaturation, for n-butanoate and longer FAAs, are linear with n-alkyl chain length whereas those for formate, acetate, and n-propionate deviate from linearity; those for acetate and n-propionate are even greater than that of n-butanoate, thereby suggesting, in addition to the common class of sites available to all such ligands, the presence of an additional class of lower affinity binding sites available only to these shortest ligands. Competition experiments involving acetate and n-octanoate and involving n-pentanoate and n-octanoate confirmed the binding of acetate to lower affinity sites unavailable to n-octanoate and n-pentanoate. Furthermore, an equation is provided, allowing computation of the transition temperature as a function of the free energy for any reversible process causing a change in thermal stability of a protein undergoing reversible, two-state denaturation. With this equation, modeling the competition experiments by using the binding parameters determined by DSC provides additional support for the class of lower affinity sites, which play a significant role in thermal stabilization of albumin at higher concentrations of these shortest FAAs.

Singh R., Haque I., and Ahmad F. (2005) Counteracting osmolyte trimethylamine N-oxide destabilizes proteins at pH below its pKa. Measurements of thermodynamic parameters of proteins in the presence and absence of trimethylamine N-oxide. *J Biol Chem.* **280**, 11035-42.

Abstract: Earlier studies have reported that trimethylamine N-oxide (TMAO), a naturally occurring osmolyte, is a universal stabilizer of proteins because it folds unstructured proteins and counteracts the deleterious effects of urea and salts on the structure and function of proteins. This conclusion has been reached from the studies of the effect of TMAO on proteins in the pH range 6.0-8.0. In this pH range TMAO is almost neutral (zwitterionic form), for it has a pK(a) of 4.66 +/- 0.10. We have asked the question of whether the effect of TMAO on protein stability is pH-dependent. To answer this question we have carried out thermal denaturation studies of lysozyme, ribonuclease-A, and apo-alpha-lactalbumin in the presence of various TMAO concentrations at different pH values above and below the pK(a) of TMAO. The main conclusion of this study is that near room temperature TMAO destabilizes proteins at pH values below its pK(a), whereas it stabilizes proteins at pH values above its pK(a). This conclusion was reached by determining the T(m) (midpoint of denaturation), delta H(m) (denaturational enthalpy change at T(m)), delta C(p) (constant pressure heat capacity change), and delta G(D) degrees (denaturational Gibbs energy change at 25 degrees C) of proteins in the presence of different TMAO concentrations. Other conclusions of this study are that T(m) and delta G(D) degrees depend on TMAO concentration at each pH value and that delta H(m) and the delta C(p) are not significantly changed in presence of TMAO.

Singh S. K. and Kishore N. (2006) Thermodynamic insights into the binding of Triton X-100 to globular proteins: a calorimetric and spectroscopic investigation. *J Phys Chem B Condens Matter Mater Surf Interfaces Biophys* **110**, 9728-9737.

Abstract: The interaction of the nonionic surfactant Triton X-100 (TX-100) with two proteins (bovine serum albumin (BSA) and alpha-lactalbumin (alpha-LA)) has been investigated by using a combination of differential scanning calorimetry, isothermal titration calorimetry, and fluorescence and circular dichroism spectroscopies. All of the calorimetric transitions in BSA were partially reversible, while being two-state and reversible in the case of alpha-LA. TX-100 molecules do not reduce the thermal stability of the protein in the monomeric form. However, in the micellar form the protein might become thermally destabilized by the micelles depending upon the nature of the protein. Isothermal titration calorimetry has been used to demonstrate that TX-100 binds to BSA at two sets of sites with 4:1 stoichiometry in each case. The van't Hoff enthalpy calculated from the temperature dependence of the binding constant did not match with the calorimetric enthalpy indicating conformational change in the protein upon surfactant binding. The surfactant binds to alpha-LA with one class of binding site, and the thermal unfolding results indicate it to be a stronger destabilizer than BSA. The fluorescence, circular dichroism, and differential scanning calorimetric results corroborate well with each other. The effect of ionic strength on the binding parameters suggests that TX-100 can bind to the protein surface via both hydrophobic and polar interactions depending upon the nature of the protein. The physical chemistry underlying the interactions between TX-100 and proteins has been presented. The mode of interaction of TX-100 with proteins is via direct binding, which has been discussed quantitatively in this work.

Supangat S., Park S. O., Seo K. H., Lee S. Y., Park Y. S. and Lee K. H. (2008) Role of Phe-99 and Trp-196 of sepiapterin reductase from *Chlorobium tepidum* in the production of L-threo-tetrahydrobiopterin. *Acta Biochim Biophys Sin. (Shanghai)* **40**, 513-518.

Abstract: Sepiapterin reductase from *Chlorobium tepidum* (cSR) catalyzes the synthesis of a distinct tetrahydrobiopterin (BH4), L-threo-BH4, different from the mammalian enzyme product. The 3-D crystal structure of cSR has revealed that the product configuration is determined solely by the substrate binding mode within the well-conserved catalytic triads. In cSR, the sepiapterin is stacked between two aromatic side chains of Phe-99 and Trp-196 and rotated approximately 180 degrees C around the active site from the position in mouse sepiapterin reductase. To confirm their roles in substrate binding, we mutated Phe-99 and/or Trp-196 to alanine (F99A, W196A) by site-directed mutagenesis and comparatively examined substrate binding of the purified proteins by kinetics analysis and differential scanning calorimetry. These mutants had higher Km values than the wild type. Remarkably, the W196A mutation resulted in a higher Km increase compared with the F99A mutation. Consistent with the results, the melting temperature (Tm) in the presence of sepiapterin was lower in the mutant proteins and the worst was W196A. These findings

indicate that the two residues are indispensable for substrate binding in cSR, and Trp-196 is more important than Phe-99 for different stereoisomer production

Surolia A., Swaminathan C. P., Ramkumar R., and Podder S. K. (1997) Unusual structural stability and ligand induced alterations in oligomerization of a galectin. *FEBS Lett* **409**, 417-420.

Abstract: L-14, a 14-kDa S-type lectin shows the jelly roll tertiary structural fold akin to legume lectins yet, unlike them, it does not dissociate on thermal unfolding. In the absence of ligand L-14 displays denaturation transitions corresponding to tetrameric and octameric entities. The presence of complementary ligand reduces the association of L-14, which is in stark contrast with legume lectins where no alterations in quaternary structures are brought about by saccharides. From the magnitude of the increase in denaturation temperature induced by disaccharides the binding constants calculated from differential scanning calorimetry are comparable with those extrapolated from titration calorimetry indicating that L-14 interacts with ligands essentially in the folded state.

Tanaka Y., Morikawa K., Ohki Y., Yao M., Tsumoto K., Watanabe N., Ohta T., and Tanaka I. (2007) Structural and mutational analyses of Drp35 from *Staphylococcus aureus*: A possible mechanism for its lactonase activity. *J Biol Chem* **282**, 5770-5780.

Abstract: Drp35 is a protein induced by cell wall-affecting antibiotics or detergents, which possesses calcium-dependent lactonase activity. To determine the molecular basis of the lactonase activity, we first solved the crystal structures of Drp35 with and without Ca(2+), which showed that the molecule has a six-bladed beta-propeller structure with two calcium ions bound at the center of the beta-propeller and surface region. Mutational analyses on evolutionarily conserved residues revealed that the central calcium-binding site is critical for the enzymatic activity of Drp35. Substitution of some other amino acid residues for the calcium-binding residues demonstrated the critical contributions of Glu48, Asp138, and Asp236 to the enzymatic activity. Differential scanning calorimetric analysis revealed that the loss of activity of E48Q and D236N, but not D138N, was attributed to their inability to hold the calcium ion. Further structural analysis of the D138N mutant indicated that it lacks a water molecule bound to the calcium ion, rather than the calcium ion itself. Based on these observations and structural information, a possible catalytic mechanism in which the calcium ion and its binding residues play direct roles was proposed for the lactonase activity of Drp35.

Tanner J. W., Liebman P. A., and Eckenhoff R. G. (1998) Volatile anesthetics alter protein stability. *Toxicol Lett* **100-101**, 387-391.

Abstract: 1. We have used differential scanning calorimetry to measure the halothane induced change in stability of five lipid-free proteins in aqueous solution. 2. The temperature at peak heat capacity (T_m) as the sample is heated provides a measure of stability. 3. Addition of halothane increases T_m for bovine and human serum albumin, but decreases T_m for hen egg white lysozyme, bovine pancreatic ribonuclease A, and horse skeletal muscle myoglobin. 4. A shift of T_m in either direction may model the action of inhaled anesthetics on relevant proteins in the central nervous system.

Tanner J. W., Eckenhoff R. G., and Liebman P. A. (1999) Halothane, an inhalational anesthetic agent, increases folding stability of serum albumin. *Biochim Biophys Acta* **1430**, 46-56.

Abstract: Inhalational anesthetic agents are known to alter protein function, but the nature of the interactions underlying these effects remains poorly understood. We have used differential scanning calorimetry to study the effects of the anesthetic agent halothane on the thermally induced unfolding transition of bovine serum albumin. We find that halothane (0.6-10 mM) stabilizes the folded state of this protein, increasing its transition midpoint temperature from 62 to 71 degrees C. Binding of halothane to the native state of serum albumin thus outweighs any non-specific interactions between the thermally unfolded state of serum albumin and halothane in this concentration range. Based on the average enthalpy change ΔH for unfolding of 170 kcal/mol, the increase from 62 to 71 degrees C corresponds to an additional Gibbs energy of stabilization ($\Delta\Delta G$) due to halothane of more than 4 kcal/mol. Analysis of the dependence of $\Delta\Delta G$ on halothane concentration shows that thermal unfolding of a bovine serum albumin molecule is linked to the dissociation of about one halothane molecule at lower halothane concentrations and about six at higher halothane concentrations. Serum albumin is the first protein that has been shown to be stabilized by an inhalational anesthetic.

Tanner J. W., Johansson J. S., Liebman P. A., and Eckenhoff R. G. (2001) Predictability of weak binding from X-ray crystallography: inhaled anesthetics and myoglobin. *Biochemistry* **40**, 5075-5080.

Abstract: Xenon and dichloromethane are inhalational anesthetic agents whose binding to myoglobin has been demonstrated by X-ray crystallography. We explore the thermodynamic significance of such binding using differential scanning calorimetry, circular dichroism spectroscopy, and hydrogen-tritium exchange measurements to study the effect of these agents on myoglobin folding stability. Though specific binding of these anesthetics might be expected to stabilize myoglobin against unfolding, dichloromethane actually destabilized myoglobin at all examined concentrations of this anesthetic (15, 40, and 200 mM). On the other hand, xenon (1 atm) stabilized myoglobin. Thus, dichloromethane and xenon have opposite effects on myoglobin stability despite localization in comparably folded X-ray crystallographic structures. These results suggest a need for solution measurements to complement crystallography if the consequences of weak binding to proteins are to be appreciated.

Thompson P. A., Wang S., Howett L. J., Wang M. M., Patel R., Averill A., Showalter R. E., Li B. and Appleman J. R. (2008) Identification of ligand binding by protein stabilization: comparison of ATLAS with biophysical and enzymatic methods. *Assay Drug Dev. Technol.* **6**, 69-81.

Abstract: ATLAS (Any Target Ligand Affinity Screen) (Anadys Pharmaceuticals, Inc., San Diego, CA) is a homogeneous, affinity-based high-throughput screening technology based on protein thermal denaturation and the ability of ligands to bind and stabilize the target protein from unfolding. To further understand the assay sensitivity for the identification of ligands that bind to soluble protein targets, firefly luciferase was chosen to characterize the technology. Luciferase is a multidomain protein with a complex unfolding pathway. Binding of ATP results in a stabilizing conformational rearrangement of the domains. Using luciferase to characterize the ATLAS technology allowed us to evaluate the generality of the screening method for the identification of ligand binding to any target. Luciferase inhibitors identified from functional screens were used to assess the capability of ATLAS to rank order inhibitors. Comparison of the ATLAS 50% effective concentration with other biophysical and biochemical methods offered insight into optimizing ATLAS assay conditions to maximize sensitivity to compound binding and protein stabilization. The results show the importance of characterizing the thermal unfolding and aggregation behavior of the protein to allow the ATLAS screen to be optimally designed

Thorolfsson M., Ibarra-Molero B., Fojan P., Petersen S. B., Sanchez-Ruiz J. M., and Martinez A. (2002) L-phenylalanine binding and domain organization in human phenylalanine hydroxylase: a differential scanning calorimetry study. *Biochemistry* **41**, 7573-7585.

Abstract: Human phenylalanine hydroxylase (hPAH) is a tetrameric enzyme that catalyzes the hydroxylation of L-phenylalanine (L-Phe) to L-tyrosine; a dysfunction of this enzyme causes phenylketonuria. Each subunit in hPAH contains an N-terminal regulatory domain (Ser2-Ser110), a catalytic domain (Asp112-Arg410), and an oligomerization domain (Ser411-Lys452) including dimerization and tetramerization motifs. Two partially overlapping transitions are seen in differential scanning calorimetry (DSC) thermograms for wild-type hPAH in 0.1 M Na-Hepes buffer, 0.1 M NaCl, pH 7.0. Although these transitions are irreversible, studies on their scan-rate dependence support that the equilibrium thermodynamics analysis is permissible in this case. Comparison with the DSC thermograms for truncated forms of the enzyme, studies on the protein and L-Phe concentration effects on the transitions, and structure-energetic calculations based on a modeled structure support that the thermal denaturation of hPAH occurs in three stages: (i) unfolding of the four regulatory domains, which is responsible for the low-temperature calorimetric transition; (ii) unfolding of two (out of the four) catalytic domains, which is responsible for the high-temperature transition; and (iii) irreversible protein denaturation, which is likely responsible for the observed exothermic distortion in the high-temperature side of the high-temperature transition. Stages 1 and 2 do not appear to be two-state processes. We present an approach to the analysis of ligand effects on DSC transition temperatures, which is based on the general binding polynomial formalism and is not restricted to two-state transitions. Application of this approach to the L-Phe effect on the DSC thermograms for hPAH suggests that (i) there are no binding sites for L-Phe in the regulatory domains; therefore, contrary to the common belief, the activation of PAH by L-Phe seems to be the result of its homotropic cooperative binding to the active sites. (ii) The regulatory domain appears to be involved in cooperativity through its interactions with the catalytic and oligomerization domains; thus, upon regulatory domain unfolding, the cooperativity in the binding of L-Phe to the catalytic domains seems to be lost and the value of the L-Phe concentration corresponding to half-saturation is increased. Overall, our results

contribute to the understanding of the conformational stability and the substrate-induced cooperative activation of this important enzyme.

Todd M. J. and Freire E. (1999) The effect of inhibitor binding on the structural stability and cooperativity of the HIV-1 protease. *Proteins* **36**, 147-156.

Abstract: The effects of the peptide inhibitor acetyl pepstatin on the structural stability of the HIV-1 protease have been measured by high sensitivity calorimetric techniques. At 25 degrees C and pH 3.6, acetyl pepstatin binds to HIV-1 protease with an affinity of $1.6 \times 10^7 \text{ M}^{-1}$ and an enthalpy of 7.3 ± 0.5 kcal/mol, indicating that binding is not favored enthalpically and that the favorable Gibbs energy originates from a large positive entropy. Since the binding of acetyl pepstatin is associated with a negative change in heat capacity ($-450 \text{ cal/K}\cdot\text{mol}$) the association reaction becomes enthalpically favored at temperatures higher than 40 degrees C. The presence of the inhibitor stabilizes the dimeric structure of the protease in a fashion that can be quantitatively described by a set of thermodynamic linkage equations. The combination of titration and differential scanning calorimetry provides an accurate way of determining binding constants for high affinity inhibitors that cannot be determined by titration calorimetry alone. A structure-based thermodynamic analysis of the binding process indicates that the stabilization effect is not distributed uniformly throughout the protease molecule. The binding of the inhibitor selectively stabilizes those conformational states in which the binding site is formed, triggering a redistribution of the state probabilities in the ensemble of conformations populated under native conditions. As a result, the stability constants for individual residues do not exhibit the same change in magnitude upon inhibitor binding. Residues in certain areas of the protein are affected significantly whereas residues in other areas are not affected at all. In particular, inhibitor binding has a significant effect on those regions that define the binding site, especially the flap region which becomes structurally stable as a result of the additional binding free energy. The induced stabilization propagates to regions not in direct contact with the inhibitor, particularly to the strand between residues Pro9 and Ala22 and the helix between Arg87 and Gly94. On the other hand, the stability of the strand between Asp60 and Leu76 is not significantly affected by inhibitor binding. The structural distribution of binding effects define cooperative pathways within the protease molecule. *Proteins* 1999;36:147-156.

Todorova N. A., Doseeva V., Ramprakash J. and Schwarz F. P. (2008) Effect of the distal C162S mutation on the energetics of drug binding to p38alpha MAP kinase. *Arch Biochem Biophys* **469**, 232-242.

Abstract: The binding reactions of the inhibitor drugs, SB 203580, SKF 86002, and p38 INH.1 to the isoforms 1 and 2 splice variants of p38alpha MAP kinase and their C162S mutants, as determined from ITC measurements from 25 to 35 degrees C, are totally enthalpically driven with binding constants ranging from $10(7)\text{M}(-1)$ for SKF 86002 and SB 203580 to $10(9)\text{M}(-1)$ for p38 INH.1. Interactions of p38 INH.1 with an additional hydrophobic pocket of the kinase would account for its large increase in $K(b)$. DSC scans exhibited single unfolding transitions for the isoforms, their mutants, and the mutants bound to the drug inhibitors. Two transitions, however, were observed for the isoform-drug complexes of SB 203580 and p38 INH.1 and were attributed to decoupled unfolding of the N- and C-terminal domains of the kinase. The C-terminal domain of isoform 1 is estimated to be less stable than of isoform 2 by $15 \text{ kJ mol}(-1)$

Torrecillas A., Laynez J., Menendez M., Corbalan-Garcia S., and Gomez-Fernandez J. C. (2004) Calorimetric study of the interaction of the C2 domains of classical protein kinase C isoenzymes with Ca^{2+} and phospholipids. *Biochemistry* **43**, 11727-11739.

Abstract: The affinities of Ca^{2+} and anionic lipid vesicles from the C2 domains of classical protein kinase C subfamily (alpha, betaII, and gamma) were studied using isothermal titration calorimetry (ITC). In addition, the thermal stability of these C2 domains in the presence of different ligand concentrations was analyzed using differential scanning calorimetry (DSC). These three closely related C2 domains bind Ca^{2+} in a similar way, demonstrating the presence of two sets of sites. The first set of sites binds one Ca^{2+} ion exothermically with similar high affinity for the three proteins (K_d around $1 \mu\text{M}$), while the second set of sites binds endothermically approximately two Ca^{2+} ions with lower affinity, which varies for each C2 domain: $22.2 \mu\text{M}$ for the PKCalpha-C2 domain, $17.2 \mu\text{M}$ for the PKCbetaII-C2 domain, and $4.3 \mu\text{M}$ for the PKCgamma-C2 domain. In the absence of Ca^{2+} the three C2 domains showed a weak interaction with vesicles containing anionic phospholipids. However, in the presence of a saturating Ca^{2+} concentration, the C2 domains increased their affinities for the anionic lipid vesicles. In all cases, the C2 domains bound the vesicles exothermically and with similar affinities. A DSC thermal stability study of the C2 domains in the

presence of Ca^{2+} and anionic lipids provided further information about this protein-ligand interaction. The presence of increasing Ca^{2+} concentrations was matched by an increase in the T_m in all cases, which was even greater in the presence of anionic lipid vesicles. The extent of the change in T_m differed for each C2 domain, reflecting the differing effect of the ligands bound during the protein stabilization. Denaturation of the C2 domains was irreversible both in the absence and in the presence of ligands, although the thermograms were not kinetically controlled. The dependence of the T_m on the Ca^{2+} concentration indicates that the protein stabilization observed by DSC primarily reflects the saturation by the cation of the low-affinity set of sites.

Ueda I., Suzuki A., and Kamaya H. (1998) Do anesthetics act by competitive binding to specific receptors? Phase transition of firefly luciferase. *Toxicol Lett* **100-101**, 405-411.

Abstract: 1. Firefly luciferase (FFL) emits a flash of light when mixed with ATP and luciferin in the presence of molecular oxygen. 2. Halothane inhibited FFL at 0.22 mM, while myristic acid inhibited it at 0.68 μM . Under steady-state conditions, myristic acid competed with luciferin. The Lineweaver-Burk plots of anesthetics were nonlinear. 3. The Hill numbers for anesthetics were above 1, while that of myristic acid was below 1 (0.89). Anesthetics interact with FFL cooperatively at multiple sites. The binding site of myristic acid is limited. 4. FFL undergoes a phase transition at 37-40 degrees C. Anesthetics decreased, while myristic acid increased the transition temperature. 5. The contrasting effects of anesthetics and myristic acid are discussed according to Koshland's transition-state model for myristic acid, which involves specific receptors, and Eyring's unfolded-state model for anesthetics, which involves nonspecific conformational change. Fatty acids are receptor-binders and structure-maker of FFL. Anesthetics are non-specific binders and structure-breaker of FFL.

Ueda I. and Suzuki A. (1998) Irreversible phase transition of firefly luciferase: contrasting effects of volatile anesthetics and myristic acid. *Biochim Biophys Acta* **1380**, 313-319.

Abstract: Firefly luciferase (FFL) has been used as a lipid-free protein model to study direct interaction of anesthetics with proteins. FFL emits a burst of light when luciferin and ATP are added in the presence of oxygen. Volatile anesthetics inhibited FFL at mM ranges, while myristic acid inhibited it at μM range. Despite the large difference, octanol/water partition coefficients of both myristic acid and halothane are 199. Differential scanning calorimetry (DSC) showed that thermal transition occurred at 38.5 degrees C with excess enthalpy of denaturation of 91.9 kcal mol⁻¹. The transition, however, was irreversible. According to the irreversible transition kinetics, the anesthetic effects were evaluated by the temperature where the irreversible transition is half completed ($T_{1/2}$). Volatile anesthetics decreased $T_{1/2}$ at mM ranges, while myristic acid and oxyluciferyladenylate (luciferin competitor) increased it at μM ranges. Luciferin is a heterocyclic carboxylate and acylates AMP. Carboxyl group of myristic acid appears to make a high affinity contact to the luciferin-recognition sites. The induced-fit theory states that binding of substrates induces the enzyme into high-energy transition state. Myristic acid stabilized FFL at the transition state, which resisted thermal denaturation. Anesthetics destabilized FFL by reversibly unfolding the protein into less active intermediate states and promoted irreversible transition when the temperature is elevated.

Ueda I. and Suzuki A. (1998) Is there a specific receptor for anesthetics? Contrary effects of alcohols and fatty acids on phase transition and bioluminescence of firefly luciferase. *Biophys J* **75**, 1052-1057.

Abstract: Firefly luciferase emits a burst of light when mixed with ATP and luciferin (L) in the presence of oxygen. This study compared the effects of long-chain n-alcohols (1-decanol to 1-octadecanol) and fatty acids (decanoic to octadecanoic acids) on firefly luciferase. Fatty acids were stronger inhibitors of firefly luciferase than n-alcohols. Myristyl alcohol inhibited the light intensity by 50% (IC_{50}) at 13.6 μM , whereas the IC_{50} of myristic acid was 0.68 μM . According to the Meyer-Overton rule, fatty acids are approximately 12,000-fold stronger inhibitors than corresponding alcohols. The Lineweaver-Burk plot showed that myristic acid inhibited firefly luciferase in competition with luciferin, whereas myristyl alcohol inhibited it noncompetitively. The differential scanning calorimetry (DSC) showed that an irreversible thermal transition occurred at approximately 39 degrees C with a transition ΔHcal of 1.57 cal g⁻¹. The ligand effects on the transition were evaluated by the temperature where the irreversible change is half completed. Alcohols decreased whereas fatty acids increased the thermal transition temperature of firefly luciferase. Koshland's transition-state theory (Science. 1963. 142:1533-1541) states that ligands that bind to the substrate-recognition sites induce the enzyme at a transition state, which is more stabilized than the native

state against thermal perturbation. The long-chain fatty acids bound to the luciferin recognition site and stabilized the protein conformation at the transition state, which resisted thermal denaturation. Eyring's unfolding theory (Science. 1966. 154:1609-1613) postulates that anesthetics and alcohols bind nonspecifically to interfacial areas of proteins and reversibly unfold the conformation. The present results showed that alcohols do not compete with luciferin and inhibit firefly luciferase nonspecifically by unfolding the protein. Fatty acids are receptor binders and stabilize the protein conformation at the transition state.

Van Dort H. M., Low P. S., Cordes K. A., Schopfer L. M., and Salhany J. M. (1994) Calorimetric evidence for allosteric subunit interactions associated with inhibitor binding to band 3 transporter. *J Biol Chem* **269**, 59-61.

Abstract: A calorimetric endotherm occurring at 68 degrees C (the C-transition) has been assigned previously to the integral domain of band 3 and was shown to be shifted to 78 degrees C after covalent binding of 4,4'-diisothiocyanostilbene-2,2'-disulfonate (DIDS). In this study, we correlate the fractional appearance of the shifted C-transition with the fraction of DIDS bound to the band 3 monomer population. Our results show a distinctly nonlinear correlation plot with the appearance of the shifted C-transition lagging behind DIDS labeling of the band 3 monomer population. The lag suggests that both monomers of a band 3 dimer must be labeled by DIDS in order for the shifted C-transition to appear at 78 degrees C, implying that the thermal unfolding of the integral domain of band 3 is modulated by allosteric interactions between subunits. This is the first in situ structural evidence supporting ligand-mediated subunit interactions within a "carrier"-type transporter protein oligomer.

Vidaud C., Gourion-Arsiquaud S., Rollin-Genetet F., Torne-Celer C., Plantevin S., Pible O., Berthomieu C. and Quemeneur E. (2007) Structural consequences of binding of UO₂(2+) to apotransferrin: can this protein account for entry of uranium into human cells? *Biochemistry* **46**, 2215-2226.

Abstract: It has been established that transferrin binds a variety of metals. These include toxic uranyl ions which form rather stable uranyl-transferrin derivatives. We determined the extent to which the iron binding sites might accommodate the peculiar topographic profile of the uranyl ion and the consequences of its binding on protein conformation. Indeed, metal intake via endocytosis of the transferrin/transferrin receptor depends on the adequate coordination of the metal in its site, which controls protein conformation and receptor binding. Using UV-vis and Fourier transform infrared difference spectroscopy coupled to a microdialysis system, we showed that at both metal binding sites two tyrosines are uranyl ligands, while histidine does not participate with its coordination sphere. Analysis by circular dichroism and differential scanning calorimetry (DSC) showed major differences between structural changes associated with interactions of iron or uranyl with apotransferrin. Uranyl coordination reduces the level of protein stabilization compared to iron, but this may be simply related to partial lobe closure. The lack of interaction between uranyl-TF and its receptor was shown by flow cytometry using Alexa 488-labeled holotransferrin. We propose a structural model summarizing our conclusion that the uranyl-TF complex adopts an open conformation that is not appropriate for optimal binding to the transferrin receptor.

Visegrady B., Lorinczy D., Hild G., Somogyi B., and Nyitrai M. (2005) A simple model for the cooperative stabilisation of actin filaments by phalloidin and jasplakinolide. *FEBS Lett* **579**, 6-10.

Abstract: The stabilisation of magnesium actin filaments by phalloidin and jasplakinolide was studied using the method of differential scanning calorimetry. The results showed that actin could adapt three conformations in the presence of drugs. One conformation was adapted in direct interaction with the drug, while another conformation was identical to that observed in the absence of drugs. A third conformation was induced through allosteric inter-protomer interactions. The effect of both drugs propagated cooperatively along the actin filaments. The number of the cooperative units determined by using a quantitative model was larger for jasplakinolide (15 actin protomers) than for phalloidin (7 protomers).

Waldron T. T., Schrifft G. L., and Murphy K. P. (2005) The salt-dependence of a protein-ligand interaction: ion-protein binding energetics. *J Mol Biol* **346**, 895-905.

Abstract: Using the binding of a nucleotide inhibitor (guanosine-3'-monophosphate) to a ribonuclease (ribonuclease Sa) as a model system, we show that the salt-dependence of the interaction arises due to specific ion binding at the site of nucleotide binding. The presence of specific ion-protein binding is concluded from a combination of differential scanning calorimetry and NMR data. Isothermal titration

calorimetry data are then fit to determine the energetic profile (enthalpy, entropy, and heat capacity) for both the ion-protein and nucleotide-protein interactions. The results provide insight into the energetics of charge-charge interactions, and have implications for the interpretation of an observed salt-dependence. Further, the presence of specific ion-binding leads to a system behavior as a function of temperature that is drastically different from that predicted from Poisson-Boltzmann calculations.

Waldron T. T. and Murphy K. P. (2003) Stabilization of proteins by ligand binding: application to drug screening and determination of unfolding energetics. *Biochemistry* **42**, 5058-5064.

Abstract: The observed stability of a protein is altered when ligands bind, which results in a shift in the melting temperature (T_m). Binding to the native state in the absence of binding to the denatured state will necessarily lead to an increase in the T_m , while binding to the unfolded state in the absence of native state binding will decrease the T_m relative to that of the protein in the absence of ligand. These effects are required by the thermodynamics of reversible folding. However, the relationship between binding affinity and the magnitude of the observed temperature shift is not a simple correlation (i.e., a larger shift in T_m does not necessarily mean tighter binding) and is complicated by interaction with the denatured state. Using exact simulations, the range of behavior for the dependence of the observed T_m shift on the energetics of ligand binding is investigated here. Specifically, differential scanning calorimetry (DSC) curves are simulated for protein unfolding in the presence of ligands binding to both the native and denatured states. The results have implications for drug screening and the determination of heat capacity changes for protein unfolding.

Wasylewski M. (2000) Binding study of riboflavin-binding protein with riboflavin and its analogues by differential scanning calorimetry. *J Protein Chem* **19**, 523-528.

Abstract: Thermal unfolding parameters of hens' egg-white riboflavin-binding-protein (RBP) were measured by differential scanning calorimetry. Thermal denaturation scans of apoRBP and RBP complexes with riboflavin and its analogues (FMN, N10 DL-glyceryl isoalloxazine, and N10 omega-hydroxy-pentyl isoalloxazine) have been measured. It was found that ligand binding causes increase of RBP thermal stability, as manifested by a change of denaturation temperature from 60.8 degrees C for apoRBP to 72.8 degrees C for RBP-Rf complex. For RBP-FMN complex, the denaturation temperature of 73.0 degrees C was even higher than for the RBP-Rf complex. The other two flavin analogues showed transition temperatures in between 66.9 degrees C and 68.8 degrees C, respectively. Analysis of excess heat capacity data showed that the best fit was the sum of two independent thermal transitions. One of the transitions, which contributed approximately 70% to the total heat effect, has transition temperature in the broad range of 60.5-73.2 degrees C; the other transition temperature is in the narrower range of 65.4-71.1 degrees C. The observed transitions can be related to RBP domains.