

DSC V - Protein aggregation studies

Ahrer K., Buchacher A., Iberer G., and Jungbauer A. (2006) Thermodynamic stability and formation of aggregates of human immunoglobulin G characterised by differential scanning calorimetry and dynamic light scattering. *J Biochem Biophys Methods* **66**, 73-86.

Abstract: The final process step of polyclonal human immunoglobulin G is formulation with agents such as sugars, polyols, amino acid and salts. Often the most stable formulations were empirically identified. Physicochemical methods, such as differential scanning calorimetry and dynamic light scattering, provide a deeper insight on the biophysical properties of such a protein solution. The combination of these methods proved to be sensitive enough to detect fine differences in the properties relevant for the development of stable protein solutions. The influence of additives, such as maltose and glycine in combination with water or low concentrations of salts, on human immunoglobulin preparations was analysed. Differential scanning calorimetry illustrated that 0.2 M glycine had better stabilising effects compared to 10% maltose. Dynamic light scattering and differential scanning calorimetry revealed that solutions preventing aggregation were not optimal in terms of thermodynamic stability. Aggregation was minimised with increasing ionic strength, shown by dynamic light scattering, whereas thermodynamic stability for heat sensitive parts of human immunoglobulin G, analysed with differential scanning calorimetry, was decreased.

Andrews J. M. and Roberts C. J. (2007) Non-native aggregation of alpha-chymotrypsinogen occurs through nucleation and growth with competing nucleus sizes and negative activation energies. *Biochemistry* **46**, 7558-7571.

Abstract: The kinetics and structural transitions of non-native aggregation of alpha-chymotrypsinogen (aCgn) were investigated over a wide range of temperature and initial protein concentration at pH 3.5, where high molecular weight aggregates remained soluble throughout the reaction. A comparison of thermodynamic, kinetic, and spectroscopic data shows that aggregation under non-native-favoring conditions proceeds through a molten globule unfolded monomer state, with a nucleation and growth mechanism. Formation of irreversible aggregates and conversion to beta-sheet secondary structures occur simultaneously without detectable intermediates, suggesting that beta-sheet formation may be a commitment step during the nucleation and growth stages. Analysis of the kinetics using a Lumry-Eyring with nucleated polymerization (LENP) model provides the predominant nucleus size and the product of the intrinsic nucleation and intrinsic growth time scales at each state point. We find that the nucleus size depends on both temperature and protein concentration, and in some cases there is competition between two distinct nucleus sizes. The observed rate coefficient (k_{obs}) for aggregation displays a maximum as a function of temperature because of the competition between folding-unfolding thermodynamics and the intrinsic growth and nucleation rates; the latter contribution has a large, negative activation enthalpy that dominates k_{obs} at elevated temperatures. Temperature-jump experiments reveal that aggregates depolymerize at high temperatures, indicating that they are lower in enthalpy than the free monomer. Overall, the results suggest more generally that non-native aggregation may proceed through more than one nucleus size and that intrinsic kinetics of nucleation and growth may have significant entropic barriers,

Antonov Y. A. and Wolf B. A. (2005) Calorimetric and structural investigation of the interaction between bovine serum albumin and high molecular weight dextran in water. *Biomacromolecules* **6**, 2980-2989.

Abstract: This work studies specific interactions between a small globular protein and a highly flexible, branched polysaccharide using differential scanning calorimetry (DSC), circular dichroism (CD), fluorescence, and turbidimetry measurements. It uses the system water/bovine serum albumin (BSA)/dextran (D 2000) as a model. Dextran molecules are able to form interpolymeric complexes with BSA in water at both low and high temperatures if the polysaccharide is in excess and if the protein exists in its associated state. It leads to a partial destabilization of the secondary and tertiary structures of the protein and an additional exposure of the hydrophobic tryptophan residues to the surface of globule. If the total concentration of biopolymers in the mixture is high enough, the stability of the protein molecules with respect to unfolding and thermoaggregation is significantly decreased as a result of an increase in the protein hydrophobicity.

Arai S. and Hirai M. (1999) Reversibility and hierarchy of thermal transition of hen egg-white lysozyme studied by small-angle x-ray scattering. *Biophys J* **76**, 2192-2197.

Abstract: To clarify mechanisms of folding and unfolding of proteins, many studies of thermal denaturation of proteins have been carried out at low protein concentrations because in many cases thermal denaturation accompanies a great tendency of aggregation. As small-angle x-ray scattering (SAXS) measurements are liable to use low-concentration solutions of proteins to avoid aggregation, SAXS has been regarded as very difficult to observe detailed features of thermal structural transitions such as intramolecular structural changes. By using synchrotron radiation SAXS, we have found that the presence of repulsive interparticle interaction between proteins can maintain solute particles separately to prevent further aggregation in thermal denaturation processes and that under such conditions the thermal structural transition of hen egg-white lysozyme (HEWL) holds high reversibility even at 5% w/v HEWL below pH approximately 5. Because of the use of the high concentration of the solutions, the scattering data has enough high-statistical accuracy to discuss the thermal structural transition depending on the structural hierarchy. Thus, the tertiary structural change of HEWL starts from mostly the onset temperature determined by the differential scanning calorimetry measurement, which accompanies a large heat absorption, whereas the intramolecular structural change, corresponding to the interdomain correlation and polypeptide chain arrangement, starts much prior to the above main transition. The present finding of the reversible thermal structural transitions at the high protein concentration is expected to enable us to analyze multiplicity of folding and unfolding processes of proteins in thermal structural transitions.

Azuaga A. I., Dobson C. M., Mateo P. L., and Conejero-Lara F. (2002) Unfolding and aggregation during the thermal denaturation of streptokinase. *Eur J Biochem* **269**, 4121-4133.

Abstract: The thermal denaturation of streptokinase from *Streptococcus equisimilis* (SK) together with that of a set of fragments encompassing each of its three domains has been investigated using differential scanning calorimetry (DSC). Analysis of the effects of pH, sample concentration and heating rates on the DSC thermograms has allowed us to find conditions where thermal unfolding occurs unequivocally under equilibrium. Under these conditions, pH 7.0 and a sample concentration of less than approximately 1.5 mg x mL⁻¹, or pH 8.0, the heat capacity curves of intact SK can be quantitatively described by three independent two-state transitions, each of which compares well with the two-state transition observed for the corresponding isolated SK domain. The results indicate that each structural domain of SK behaves as a single cooperative unfolding unit under equilibrium conditions. At pH 7.0 and high sample concentration, or at pH 6.0 at any concentration investigated, the thermal unfolding of domain A was accompanied by the time-dependent formation of aggregates of SK. This produces a severe deformation of the DSC curves, which become concentration dependent and kinetically controlled, and thus precludes their proper analysis by standard deconvolution methods. A simple model involving time-dependent, high-order aggregation may account for the observed effects. Limited-proteolysis experiments suggest that in the aggregates the N-terminal segment 1-63 and the whole of SK domain C are at least partially structured, while domain B is highly unstructured. Unfolding of domain A, under conditions where the N-terminal segment 1-63 has a high propensity for beta sheet structure and a partially formed hydrophobic core, gives rise to rapid aggregation. It is likely that this region is able to act as a nucleus for the aggregation of the full-length protein.

Babu K. R. and Bhakuni V. (1997) Ionic-strength-dependent transition of hen egg-white lysozyme at low pH to a compact state and its aggregation on thermal denaturation. *Eur J Biochem* **245**, 781-789.

Abstract: Equilibrium acid-induced unfolding of hen egg-white lysozyme has been investigated by a combination of optical methods, size-exclusion chromatography, and differential scanning calorimetry. The results showed the presence of a partially folded state of hen egg-white lysozyme at pH 1.5, characterized by a substantial secondary structure, a large solvent exposure of non-polar clusters, and significantly disrupted tertiary structure. A large enthalpy was also associated with the conversion of the acid-unfolded state to a fully unfolded state. Size-exclusion chromatography and 8-anilino-1-naphthalenesulphonic acid-binding studies showed an ionic-strength-induced transition of the partially folded state to a compact conformation. Furthermore, an ionic-strength-dependent aggregation on thermal unfolding of the partially folded intermediate was also observed. These observations provide insights into the possible features responsible for the stabilization of intermediates in the folding of hen egg-white lysozyme.

Backmann J., Schafer G., Wyns L., and Bonisch H. (1998) Thermodynamics and kinetics of unfolding of the thermostable trimeric adenylate kinase from the archaeon *Sulfolobus acidocaldarius*. *J Mol Biol* **284**, 817-833.

Abstract: The thermal stability of adenylate kinase from the thermoacidophilic archaeon *Sulfolobus acidocaldarius* was characterized comprehensively using denaturant-induced unfolding, differential scanning calorimetry, circular dichroism spectroscopy, and enzymological inactivation studies. The thermally induced unfolding of the protein is irreversible due to aggregation, whereas the unfolding induced by guanidinium chloride is reversible. The protein is known to be a homotrimer in its native state and we established that it unfolds upon dissociation in the case of denaturant unfolding. We measured the thermodynamic stability of the protein in a temperature range from 5 to 70 degrees C using denaturant unfolding. The protein has a maximum of stability (intrinsic free energy) of 31 kcal/mol-trimer (130 kJ/mol-trimer) at 32 degrees C (based on the linear extrapolation model). The heat capacity change upon unfolding ΔC_p and the m -value were considered to be constant in this temperature range and calculated to be 2.86 kcal/mol-trimer (11.9 kJ/mol-trimer) and 5.67 kcal/mol-trimer M (23.7 kJ/mol-trimer M), respectively. The influence of trimerization on thermodynamic stability was investigated. The several interrelated aspects of thermal stability such as unfolding kinetics, the temperature-dependence of the free energy, and the concentration and temperature-dependencies of the fraction of denatured protein are described quantitatively. The properties of the Gibbs-Helmholtz function of the adenylate kinase from *S. acidocaldarius*, in particular, and of oligomeric proteins, in general terms, are discussed and compared with the properties of the analogous function for monomeric proteins. Moreover, we discuss methodological aspects: we obtained the analytical expression of the denaturant-unfolding isotherm for homotrimeric proteins; we include a formula Appendix containing the derivations of the expressions used.

Bagger H. L., Ogendal L. H. and Westh P. (2007) Solute effects on the irreversible aggregation of serum albumin. *Biophys Chem* **130**, 17-25.

Abstract: Thermal stress on bovine serum albumin (BSA) promotes protein aggregation through the formation of intermolecular beta-sheets. We have used light scattering and chromatography to study effects of (<1 M) Na₂SO₄, NaSCN, sucrose, sorbitol and urea on the rate of the thermal aggregation. Both salts were strong inhibitors of BSA aggregation and they reduced both the size and number (concentration) of aggregate particles compared to non-ionic solutes (or pure buffer). Hence, the salts appear to suppress both nucleation- and growth rate. The non-electrolyte additives reduced the initial aggregation rate (compared to pure buffer), but did not significantly limit the extent of aggregation in samples quenched after 27 min. heat exposure (40-50% aggregation in all samples). The non-electrolytes did, however, modify the aggregation process as they consistently brought about smaller but more concentrated aggregates than pure buffer. The results are discussed along the lines of linkage- and transition state theories. In this framework, the rate of the aggregation process is governed by the equilibrium between a thermally denatured state (D) and the transition state D[‡] (not equal). Thus, the effect of a solute relies on its preferential interactions with respectively D and D[‡] (not equal). The current results do not show any correlation between the solutes' preferential interactions with native BSA and their effect on the rate of aggregation. This suggests that non-specific, "Hofmeister-type" interactions, which scale with the solvent accessible surface area, are of minor importance. Rather, salt induced suppression of aggregation is suggested to depend on the modulation of specific electrostatic forces in the D[‡] (not equal) state.

Bajaj K., Chakshumathi G., Bachhawat-Sikder K., Surolia A., and Varadarajan R. (2004) Thermodynamic characterization of monomeric and dimeric forms of CcdB (controller of cell division or death B protein). *Biochem J* **380**, 409-417.

Abstract: The protein CcdB (controller of cell division or death B) is an F-plasmid-encoded toxin that acts as an inhibitor of *Escherichia coli* DNA gyrase. The stability and aggregation state of CcdB have been characterized as a function of pH and temperature. Size-exclusion chromatography revealed that the protein is a dimer at pH 7.0, but a monomer at pH 4.0. CD analysis and fluorescence spectroscopy showed that the monomer is well folded, and has similar tertiary structure to the dimer. Hence intersubunit interactions are not required for folding of individual subunits. The stability of both forms was characterized by isothermal denaturant unfolding and calorimetry. The free energies of unfolding were found to be 9.2 kcal x mol⁻¹ (1 cal approximately 4.184 J) and 21 kcal x mol⁻¹ at 298 K for the monomer and dimer respectively. The denaturant concentration at which one-half of the protein molecules are unfolded (C_m) of the dimer is dependent on protein concentration, whereas the C_m of the monomer is independent of protein concentration, as expected. Although thermal unfolding of the protein in aqueous solution is irreversible at neutral pH, it was found that thermal unfolding is reversible in the presence of GdmCl (guanidinium chloride). Differential scanning calorimetry in the presence of low concentrations of GdmCl in combination

with isothermal denaturation melts as a function of temperature were used to derive the stability curve for the protein. The value of ΔC_p (representing the change in excess heat capacity upon protein denaturation) is $2.8 \pm 0.2 \text{ kcal} \times \text{mol}^{-1} \times \text{K}^{-1}$ for unfolding of dimeric CcdB, and only has a weak dependence on denaturant concentration.

Bam N. B., Cleland J. L., Yang J., Manning M. C., Carpenter J. F., Kelley R. F., and Randolph T. W. (1998) Tween protects recombinant human growth hormone against agitation-induced damage via hydrophobic interactions. *J Pharm Sci* **87**, 1554-1559.

Abstract: In the absence of surfactants, recombinant human growth hormone (rhGH) rapidly forms insoluble aggregates during agitation. The nonionic surfactant Tween 20, when present at Tween:protein molar ratios >4 , effectively inhibits this aggregation. Differential scanning calorimetry (DSC) of rhGH solutions showed melting transitions that decreased by ca. 2 degrees C in the presence of Tween. Circular dichroism (CD) studies of the same thermal transition showed that the decrease is specific to the relatively high protein concentrations required for DSC. CD studies showed melting transitions that decreased with lower protein concentrations. Tween has an insignificant effect on the melting transition of rhGH at lower protein concentrations (0.18 mg/mL). Injection titration microcalorimetry showed that the interaction of Tween with rhGH is characterized by a weak enthalpy of binding. For comparison, interferon-g, another protein which has been shown to bind Tween, also shows weak enthalpy of binding. Fluorescent probe binding studies and infrared spectroscopic investigations of rhGH secondary structure support suggestions in the literature (Bam, N. B.; Cleland, J. L., Randolph, T. W. Molten globule intermediate of recombinant human growth hormone: stabilization with surfactants. *Biotechnol. Prog.* 1996. 12, 801-809) that Tween binding is driven by hydrophobic interactions, with little perturbation of protein secondary structure.

Beldarrain A., Cruz Y., Cruz O., Navarro M., and Gil M. (2001) Purification and conformational properties of a human interferon alpha2b produced in Escherichia coli. *Biotechnol Appl Biochem* **33**, 173-182.

Abstract: Recombinant human interferon alpha2b was expressed intracellularly in Escherichia coli as insoluble aggregates using a new expression vector, and was purified to homogeneity using essentially two-step chromatographic procedures, i.e. immobilized metal-ion-affinity chromatography and reversed-phase HPLC. The established purification process is highly reproducible and leads to a total recovery of approx. 12% with a specific biological activity of higher than 1×10^8 i.u./mg of protein, which is comparable with the international requirement for interferon alpha2b. For purified protein we report conformational stability as a function of pH and temperature using differential scanning calorimetry and CD. Thermal unfolding as a function of pH showed only one endotherm at a temperature higher than 45 degrees C, and was reversible at pH 2-3.75 and irreversible at pH 4-10. At pH 7.0, the most stable condition, the conformational stability depends on protein concentration and ionic strength. The highly helical secondary structure is very conserved over the whole pH range studied, including at high temperatures.

Beldarrain A., Acosta N., Betancourt L., Gonzalez L. J., and Pons T. (2003) Enzymic, spectroscopic and calorimetric studies of a recombinant dextranase expressed in Pichia pastoris. *Biotechnol Appl Biochem* **38**, 211-221.

Abstract: Conformational stability and structural characterization of an rDex (recombinant dextranase) expressed in Pichia pastoris were studied by enzymic assays, fluorescence, CD and DSC (differential scanning calorimetry). We also identified two disulphide bridges (Cys(9)-Cys(14), Cys(484)-Cys(488)) and two free Cys residues (Cys(336), Cys(415)) that are not conserved between bacterial and fungal dextranases of GH-49 (glycoside hydrolase family 49) by MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS. Enzymic and fluorescence studies revealed that rDex is biological and conformationally stable at acidic pH, with maximum activity at pH 4.5-5.0, while CD spectra indicated a secondary structure basically composed of beta-sheets. rDex loses biological activity at neutral pH without total disruption of its conformation. In addition, rDex preserves its conformation close to 60 degrees C, but it is thermally denatured with appreciable aggregation at temperatures above 75 degrees C. DSC studies always displayed irreversible transitions and a strong dependence on the scan rate. Our combined analysis suggested that the denaturation process of rDex is under kinetic control, which is described reasonably well by the two-state kinetic scheme.

Benitez-Cardoza C. G., Rojo-Dominguez A., and Hernandez-Arana A. (2001) Temperature-induced denaturation and renaturation of triosephosphate isomerase from Saccharomyces cerevisiae: evidence of

dimerization coupled to refolding of the thermally unfolded protein. *Biochemistry* **40**, 9049-9058.

Abstract: The thermal denaturation of the dimeric enzyme triosephosphate isomerase (TIM) from *Saccharomyces cerevisiae* was studied by spectroscopic and calorimetric methods. At low protein concentration the structural transition proved to be reversible in thermal scannings conducted at a rate greater than 1.0 degrees C min⁻¹. Under these conditions, however, the denaturation-renaturation cycle exhibited marked hysteresis. The use of lower scanning rates lead to pronounced irreversibility. Kinetic studies indicated that denaturation of the enzyme likely consists of an initial first-order reaction that forms thermally unfolded (U) TIM, followed by irreversibility-inducing reactions which are probably linked to aggregation of the unfolded protein. As judged from CD measurements, U possesses residual secondary structure but lacks most of the tertiary interactions present in native TIM. Furthermore, the large increment in heat capacity upon denaturation suggests that extensive exposure of surface area occurs when U is formed. Above 63 degrees C, reactions leading to irreversibility were much slower than the unfolding process; as a result, U was sufficiently long-lived as to allow an investigation of its refolding kinetics. We found that U transforms into natively like TIM through a second-order reaction in which association is coupled to the regain of secondary structure. The rate constants for unfolding and refolding of TIM displayed temperature dependences resembling those reported for monomeric proteins but with considerably larger activation enthalpies. Such large temperature dependences seem to be determinant for the occurrence of kinetically controlled transitions and thus constitute a simple explanation for the hysteresis observed in thermal scannings.

Benjwal S., Verma S., Rohm K. H., and Gursky O. (2006) Monitoring protein aggregation during thermal unfolding in circular dichroism experiments. *Protein Sci* **15**, 635-639.

Abstract: Thermal unfolding monitored by spectroscopy or calorimetry is widely used to determine protein stability. Equilibrium thermodynamic analysis of such unfolding is often hampered by its irreversibility, which usually results from aggregation of thermally denatured protein. In addition, heat-induced protein misfolding and aggregation often lead to formation of amyloid-like structures. We propose a convenient method to monitor in real time protein aggregation during thermal folding/ unfolding transition by recording turbidity or 90 degrees light scattering data in circular dichroism (CD) spectroscopic experiments. Since the measurements of turbidity and 90 degrees light scattering can be done simultaneously with far- or near-UV CD data collection, they require no additional time or sample and can be directly correlated with the protein conformational changes monitored by CD. The results can provide useful insights into the origins of irreversible conformational changes and test the linkage between protein unfolding or misfolding and aggregation in various macromolecular systems, including globular proteins and protein-lipid complexes described in this study, as well as a wide range of amyloid-forming proteins and peptides.

Bergamini C. M., Dean M., Matteucci G., Hanau S., Tanfani F., Ferrari C., Boggian M., and Scatturin A. (1999) Conformational stability of human erythrocyte transglutaminase. Patterns of thermal unfolding at acid and alkaline pH. *Eur J Biochem* **266**, 575-582.

Abstract: Tissue-type transglutaminase is irreversibly inactivated during heat treatment. The rate of inactivation is low at pH 7.5; it increases slightly at acid pH (6.1) but much more at alkaline pH (9.0-9.5), suggesting that specific effects take place in the alkaline range, possibly in relation to decreased stability of the transition-state intermediate as pH is raised above 9.0. Differential scanning calorimetry experiments indicate that thermal unfolding of the protein occurs with two separate transitions, involving independent regions of the enzyme. They are assigned to domains 1 and 2 and domains 3 and 4, respectively, by a combination of calorimetric and spectroscopic techniques. When considering the effects of pH, we noted that transglutaminase was unfolded via different pathways at the different pH values considered. At acid pH, the whole structure of the protein was lost irreversibly, with massive aggregation. At neutral and, even more so, at alkaline pH, aggregation was absent (or very limited at high protein concentration) and the loss of secondary structure was dependent on the ionization state of crucial lysine residues. Unfolding at pH 9.5 apparently chiefly involved the N-terminal region, as testified by changes in protein intrinsic fluorescence. In addition, the C-terminal region was destabilized at each pH value tested during thermal unfolding, as shown by digestion with V8 proteinase, which is inactive on the native protein. Evidence was obtained that the N-terminal and C-terminal regions interact with each other in determining the structure of the native protein.

Bittar E. R., Caldeira F. R., Santos A. M., AR G. n., Rogana E., and Santoro M. M. (2003) Characterization of -trypsin at acid pH by differential scanning calorimetry. *Braz J Med Biol Res* **36**, 1621-1627.

Abstract: Trypsin is a serino-protease with a polypeptide chain of 223 amino acid residues and contains six disulfide bridges. It is a globular protein with a predominance of antiparallel -sheet and helix in its secondary structure and has two domains with similar structures. We assessed the stability of -trypsin in the acid pH range using microcalorimetric (differential scanning calorimetry) techniques. Protein concentrations varied in the range of 0.05 to 2.30 mg/ml. Buffer solutions of 50.0 mM -alanine and 20.0 mM CaCl₂ at different pH values (from 2.0 to 4.2) and concentrations of sorbitol (1.0 and 2.0 M), urea (0.5 M) or guanidinium hydrochloride (0.5 and 1.0 M) were used. The data suggest that we are studying the same conformational transition of the protein in all experimental situations using pH, sorbitol, urea and guanidinium hydrochloride as perturbing agents. The observed van't Hoff ratios ($\Delta H_{cal}/\Delta H_{vH}$) of 1.0 to 0.5 in the pH range of 3.2 to 4.2 suggest protein aggregation. In contrast, $\Delta H_{cal}/\Delta H_{vH}$ ratios equal to one in the pH range of 2.0 to 3.2 suggest that the protein unfolds as a monomer. At pH 3.00, -trypsin unfolded with $T_m = 54$ C and $\Delta H = 101.8$ kcal/mol, and the change in heat capacity between the native and unfolded forms of the protein (ΔC_p) was estimated to be 2.50 0.07 kcal mol⁻¹ K⁻¹. The stability of -trypsin calculated at 298 K was $\Delta G_D = 5.7$ kcal/mol at pH 3.00 and $\Delta G_D = 15.2$ kcal/mol at pH 7.00, values in the range expected for a small globular protein.

Blaber S. I., Culajay J. F., Khurana A., and Blaber M. (1999) Reversible thermal denaturation of human FGF-1 induced by low concentrations of guanidine hydrochloride. *Biophys J* **77**, 470-477.

Abstract: Human acidic fibroblast growth factor (FGF-1) is a powerful mitogen and angiogenic factor with an apparent melting temperature (T_m) in the physiological range. FGF-1 is an example of a protein that is regulated, in part, by stability-based mechanisms. For example, the low T_m of FGF-1 has been postulated to play an important role in the unusual endoplasmic reticulum-independent secretion of this growth factor. Despite the close relationship between function and stability, accurate thermodynamic parameters of unfolding for FGF-1 have been unavailable, presumably due to effects of irreversible thermal denaturation. Here we report the determination of thermodynamic parameters of unfolding (ΔH , ΔG , and ΔC_p) for FGF-1 using differential scanning calorimetry (DSC). The thermal denaturation is demonstrated to be two-state and reversible upon the addition of low concentrations of added guanidine hydrochloride (GuHCl). ΔG values from the DSC studies are in excellent agreement with values from isothermal GuHCl denaturation monitored by fluorescence and circular dichroism (CD) spectroscopy. Furthermore, the results indicate that irreversible denaturation is closely associated with the formation of an unfolding intermediate. GuHCl appears to promote reversible two-state denaturation by initially preventing aggregation of this unfolding intermediate, and at subsequently higher concentrations, by preventing formation of the intermediate.

Boudko S. P., Londer Y. Y., Letarov A. V., Sernova N. V., Engel J., and Mesyanzhinov V. V. (2002) Domain organization, folding and stability of bacteriophage T4 fibrin, a segmented coiled-coil protein. *Eur J Biochem* **269**, 833-841.

Abstract: Fibrin is a segmented coiled-coil homotrimer of the 486-residue product of phage T4 gene wac. This protein attaches to a phage particle by the N-terminal region and forms fibrous whiskers of 530 Å, which perform a chaperone function during virus assembly. The short C-terminal region has a beta-annulus-like structure. We engineered a set of fibrin deletion mutants sequentially truncated from the N-termini, and the mutants were studied by differential scanning calorimetry (DSC) and CD measurements. The analysis of DSC curves indicates that full-length fibrin exhibits three thermal-heat-absorption peaks centred at 321 K ($\Delta H=1390$ kJ x mol trimer⁻¹), at 336 K ($\Delta H=7600$ kJ x mol trimer⁻¹), and at 345 K ($\Delta H=515$ kJ x mol trimer⁻¹). These transitions were assigned to the N-terminal, segmented coiled-coil, and C-terminal functional domains, respectively. The coiled-coil region, containing 13 segments, melts cooperatively as a single domain with a mean enthalpy $\Delta H_{res}=21$ kJ x mol residue⁻¹. The ratio of $\Delta H_{vH}/\Delta H_{cal}$ for the coiled-coil part of the 120-, 182-, 258- and 281-residue per monomer mutants, truncated from the N-termini, and for full-length fibrin are 0.91, 0.88, 0.42, 0.39, and 0.13, respectively. This gives an indication of the decrease of the 'all-or-none' character of the transition with increasing protein size. The deletion of the 12-residue-long loop in the 120-residue fibrin increases the thermal stability of the coiled-coil region. According to CD data, full-length fibrin and all the mutants truncated from the N-termini refold properly after heat denaturation. In contrast, fibrin XN, which is deleted for the C-terminal domain, forms aggregates inside the cell. The XN protein can be partially refolded by dilution from urea and does

not refold after heat denaturation. These results confirm that the C-terminal domain is essential for correct fibrin assembly both in vivo and in vitro and acts as a foldon.

Boye J. I., Ismail A. A., and Alli I. (1996) Effects of physicochemical factors on the secondary structure of beta-lactoglobulin. *J Dairy Res* **63**, 97-109.

Abstract: Fourier transform infrared spectroscopy and differential scanning calorimetry were used as complementary techniques to study changes in the secondary structure of beta-lactoglobulin under various physicochemical conditions. The effects of pH (3-9), NaCl (0-2 M), and lactose, glucose and sucrose (100-500 g/l) in the temperature range 25-100 degrees C on the conformation sensitive amide I band in the i.r. spectrum of beta-lactoglobulin in D₂O solution were examined. The 1692 cm⁻¹ band in the amide I band profile had not been definitively assigned in previous studies of the i.r. spectrum of beta-lactoglobulin. The decrease in this band at ambient temperature with time or upon mild heating was attributed to slow H-D exchange, indicating that it was due to a structure buried deep within the protein. The disappearance of the 1692 cm⁻¹ band on heating was accompanied by the appearance of two bands at 1684 and 1629 cm⁻¹, assigned to beta-sheets. The 1692 cm⁻¹ band was therefore attributed to a beta-type structure. beta-Lactoglobulin showed maximum thermal stability at pH 3 and was easily denatured at pH 9. On denaturation, the protein unfolded into more extensive random coil structures at pH 9 than at pH 3. After 10 h at pH 9 (25 degrees C), beta-lactoglobulin was partly denatured. Heating to 60-80 degrees C generally resulted in the loss of secondary structure. At all pH values studied, two new bands at 1618 and 1684 cm⁻¹, characteristic of intermolecular beta-sheet structure and associated with aggregation, were observed after the initial denaturation. Differential scanning calorimetry studies indicated that the thermal stability of beta-lactoglobulin was enhanced in the presence of sugars. The Fourier transform i.r. results obtained provide evidence that sugars promoted the unfolding of beta-lactoglobulin via multiple transition pathways leading to a transition state resisting aggregation.

Brault P. A., Kariapper M. S., Pham C. V., Flowers R. A., Gunning W. T., Shah P., and Funk M. O., Jr. (2002) Protein micelles from lipoxygenase 3. *Biomacromolecules* **3**, 649-654.

Abstract: Heat-induced conformational changes in lipoxygenase 3 were characterized by differential scanning calorimetry. The positions of the observed transitions were sensitive to the composition of the buffer. In particular, lipoxygenase 3 heated in carbonate buffer at pH 8.0 formed large soluble aggregates. Variable-temperature circular dichroism revealed that the formation of the aggregates was not accompanied by the unfolding of the C-terminal domain, which is composed primarily of alpha-helix. The aggregates were investigated using size exclusion chromatography, native polyacrylamide gel electrophoresis, dynamic light scattering, and electron microscopy. The data were consistent with the formation of roughly spherical particles with an average hydrodynamic radius of 26 nm and an approximate composite molecular weight of 10,000,000 Da. To account for the formation of soluble aggregates from lipoxygenase 3, we propose that hydrophobic amino acid residues are exposed by unfolding of the N-terminal beta-barrel domain of the protein resulting in the formation of protein micelles with a hydrophilic surface composed of the C-terminal domains.

Briers Y., Lavigne R., Plessers P., Hertveldt K., Hanssens I., Engelborghs Y., and Volckaert G. (2006) Stability analysis of the bacteriophage phiKMV lysin gp36C and its putative role during infection. *Cell Mol Life Sci* **63**, 1899-1905.

Abstract: The kinetic, thermodynamic and structural stability of gp36C, the virion-associated peptidoglycan hydrolase domain of bacteriophage phiKMV, is analyzed. Recombinant gp36C is highly thermoresistant ($k = 0.595 \text{ h}^{-1}$) at 95 degrees C, but not thermostable ($T(m) = 50.2$ degrees C, $\Delta H(\text{cal}) = 6.86 \times 10^4 \text{ cal mol}^{-1}$). However, aggregation influences kinetic stability in an unusual manner since aggregation is more pronounced at 55 degrees C than at higher temperatures. Furthermore, gp36C reversibly unfolds in a two-state endothermic transition, and circular dichroism analysis shows that gp36C almost completely refolds after a 3-h heat treatment at 85 degrees C. These properties are in agreement with gp36C being part of the extensible tail which is ejected in an unfolded state during phage infection.

Brockwell D., Yu L., Cooper S., McClelland S., Cooper A., Attwood D., Gaskell S. J., and Barber J. (2001) Physicochemical consequences of the perdeuteration of glutathione S-transferase from *S. japonicum*. *Protein Sci* **10**, 572-580.

Abstract: Glutathione S-transferase (GST) from *Schistosoma japonicum* has been prepared in both normal

protiated (pGST) and fully deuteriated (dGST) form by recombinant DNA technology. Electrospray mass spectrometry showed that the level of deuteriation in dGST was 96% and was homogeneous across the sample. This result is attributed to the use of a deuterium-tolerant host *Escherichia coli* strain in the preparation of the protein. 10 heteroatom-bound deuteriums (in addition to the carbon-bound deuteriums) were resistant to exchange when dGST was incubated in protiated buffer. The physicochemical and biological properties of the two proteins were compared. dGST was relatively less stable to heat denaturation and to proteolytic cleavage than was pGST. The midpoint transition temperature for pGST was 54.9 degrees C, whereas that for dGST was 51.0 degrees C. Static light-scattering measurements revealed that the association behavior of dGST is also different from that of pGST. The perdeuteriated enzyme shows a tendency to associate into dimers of the fundamental dimer. This is in contrast with results that have been obtained for other perdeuteriated proteins in which perdeuteriation has been shown to promote dissociation of aggregates. dGST showed a similar K_m to pGST; similar results had been obtained previously with bacterial alkaline phosphatase. However, whereas the alkaline phosphatase showed a reduced rate of catalysis on deuteriation, dGST exhibited a slightly higher rate of catalysis than pGST. It is clear that the bulk substitution of deuterium for protium has significant effects on the properties of proteins. Until many more examples have been studied, it will be difficult to predict these effects for any given protein. Nevertheless, deuteriation represents an intriguing method of preparing functional analogs of recombinant proteins.

Bullock A. N., Henckel J., DeDecker B. S., Johnson C. M., Nikolova P. V., Proctor M. R., Lane D. P., and Fersht A. R. (1997) Thermodynamic stability of wild-type and mutant p53 core domain. *Proc Natl Acad Sci U S A* **94**, 14338-14342.

Abstract: Some 50% of human cancers are associated with mutations in the core domain of the tumor suppressor p53. Many mutations are thought just to destabilize the protein. To assess this and the possibility of rescue, we have set up a system to analyze the stability of the core domain and its mutants. The use of differential scanning calorimetry or spectroscopy to measure its melting temperature leads to irreversible denaturation and aggregation and so is useful as only a qualitative guide to stability. There are excellent two-state denaturation curves on the addition of urea that may be analyzed quantitatively. One Zn^{2+} ion remains tightly bound in the holo-form of p53 throughout the denaturation curve. The stability of wild type is 6.0 kcal (1 kcal = 4.18 kJ)/mol at 25 degrees C and 9.8 kcal/mol at 10 degrees C. The oncogenic mutants R175H, C242S, R248Q, R249S, and R273H are destabilized by 3.0, 2.9, 1.9, 1.9, and 0.4 kcal/mol, respectively. Under certain denaturing conditions, the wild-type domain forms an aggregate that is relatively highly fluorescent at 340 nm on excitation at 280 nm. The destabilized mutants give this fluorescence under milder denaturation conditions.

Bulone D., Martorana V., and San Biagio P. L. (2001) Effects of intermediates on aggregation of native bovine serum albumin. *Biophys Chem* **91**, 61-69.

Abstract: Protein aggregation has been recognized to be a pathological indicator for several fatal diseases, such as Alzheimer's disease, transmissible spongiform encephalopathies, Creutzfeldt-Jacob disease, etc. Aggregation usually involves conformational changes of proteins that have acquired an intermediate beta-structure-rich conformation and can occur even at low protein concentration. Recent work in our laboratory has shown that bovine serum albumin (BSA), even at low-concentration, exhibits self-association properties related to conformational changes, so providing a very convenient model system to study this class of problems. Here we report data (obtained by different experimental techniques) on a mixture of BSA in native and intermediate (beta-structure-rich) form. Results show that the interaction between the two species is responsible for a decrease in the thermodynamic stability of the solution. This occurs without requiring noticeable conformational changes of the native protein. Results presented here can provide new insight on the "protein only" hypothesis proposed for the formation of plaques involved in several neurodegenerative diseases.

Caldinelli L., Iametti S., Barbiroli A., Bonomi F., Fessas D., Molla G., Pilone M. S., and Pollegioni L. (2005) Dissecting the structural determinants of the stability of cholesterol oxidase containing covalently bound flavin. *J Biol Chem* **280**, 22572-22581.

Abstract: Cholesterol oxidase from *Brevibacterium sterolicum* is a monomeric flavoenzyme catalyzing the oxidation and isomerization of cholesterol to cholest-4-en-3-one. This protein is a class II cholesterol oxidases, with the FAD cofactor covalently linked to the enzyme through the His(69) residue. In this work,

unfolding of wild-type cholesterol oxidase was compared with that of a H69A mutant, which does not covalently bind the flavin cofactor. The two protein forms do not show significant differences in their overall topology, but the urea-induced unfolding of the H69A mutant occurred at significant lower urea concentrations than wild-type (approximately 3 versus approximately 5 M, respectively), and the mutant protein had a melting temperature approximately 10-15 degrees C lower than wild-type in thermal denaturation experiments. The different sensitivity of the various spectroscopic features used to monitor protein unfolding indicated that in both proteins a two-step (three-state) process occurs. The presence of an intermediate was more evident for the H69A mutant at 2 M urea, where catalytic activity and tertiary structure were lost, and new hydrophobic patches were exposed on the protein surface, resulting in protein aggregation. Comparative analysis of the changes occurring upon urea and thermal treatment of the wild-type and H69A protein showed a good correlation between protein instability and the elimination of the covalent link between the flavin and the protein. This covalent bond represents a structural device to modify the flavin redox potentials and stabilize the tertiary structure of cholesterol oxidase, thus pointing to a specific meaning of the flavin binding mode in enzymes that carry out the same reaction in pathogenic versus non-pathogenic bacteria.

Campanero-Rhodes M. A., Menendez M., Saiz J. L., Sanz L., Calvete J. J., and Solis D. (2006) Zinc Ions Induce the Unfolding and Self-Association of Boar Spermadhesin PSP-I, a Protein with a Single CUB Domain Architecture, and Promote Its Binding to Heparin. *Biochemistry* **45**, 8227-8235.

Abstract: Spermadhesins are a family of seminal plasma proteins composed of a single CUB domain, which appear to be involved in various aspects of the fertilization process in pigs. PSP-I and PSP-II, the most abundant porcine spermadhesins, occur in seminal plasma as noncovalent heterodimers devoid of heparin-binding capability. Of note is the stability of this dimer, which is significantly affected by physiologically relevant conditions such as Zn(2+) ions. Here, we show that PSP-I and PSP-II when separated appear to conserve the overall fold of the CUB domain observed in the crystal structure of the PSP-I/PSP-II heterodimer, as concluded from gel filtration, analytical ultracentrifugation, differential scanning calorimetry, and circular dichroism analyses. However, Zn(2+) concentrations in the range of those found in boar seminal plasma induce the unfolding and self-association of PSP-I, apparently as a consequence of the exposure of hydrophobic core residues, whereas they have no effect on PSP-II. Remarkably, Zn(2+)-denatured and self-associated (but not structured monomeric) PSP-I is retained on a heparin column, resembling the behavior of free PSP-I and homologous spermadhesins of the heparin-binding fraction of boar seminal plasma, which also exhibit different aggregation states. Thus, the modulation of the structural organization and heparin-binding ability of PSP-I by Zn(2+) might be a physiological phenomenon in seminal plasma.

Carmona P., Molina M., and Rodriguez-Casado A. (2003) Raman study of the thermal behaviour and conformational stability of basic pancreatic trypsin inhibitor. *Eur Biophys J* **32**, 137-143.

Abstract: We have studied the thermal denaturation of native basic pancreatic trypsin inhibitor (BPTI) by monitoring the Raman bands in the 4000-400 cm⁻¹ range. In agreement with results obtained by calorimetry, a cooperative melting transition is observed starting at 75 degrees C. This transition is found to involve predominantly the unfolding of helical structures accompanied by beta-aggregation, loss of hydrophobic interactions between side chains and changes in CSSC dihedral angles. However, salt bridge breaking starts near 40 degrees C, as deduced from the nu(s)(COO(-)) band and from the bands close to 1320 and 1345 cm⁻¹ which for the first time have been shown to be due largely to vibrations of the arginine guanidyl group in BPTI. The thermal stability is, hence, attributable to cooperative contributions from hydrophobic and backbone hydrogen bond interactions as well as from disulfide bonds.

Chamani J. and Heshmati M. (2008) Mechanism for stabilization of the molten globule state of papain by sodium n-alkyl sulfates: spectroscopic and calorimetric approaches. *J Colloid Interface Sci* **322**, 119-127.

Abstract: Papain exists in a molten globule (MG) state at pH 2 and in this state protein tends to aggregate in the presence of lower concentrations of guanidine hydrochloride (GuHCl). Such aggregation is prevented if low concentrations of sodium n-alkyl sulfates are also present in the buffer; in addition, stabilization of the protein is also induced. The guanidine hydrochloride and temperature-induced unfolding of papain, in the presence of n-alkyl sulfates, indicate stabilization of the protein as seen from the higher transition midpoints when monitored by fluorescence, circular dichroism, and differential scanning calorimetry. However, a similar phenomenon is not seen under neutral conditions in the presence of n-alkyl

sulfate concentrations. The effect of n-alkyl sulfates on the structure of the MG state of papain was utilized to investigate the contribution of hydrophobic interaction to the stability of the MG state. The Td values of the MG states of papain in the presence of n-alkyl sulfates at different concentrations showed substantial variation. The enhancement of Td values at the stability criterion of MG states corresponded with increasing chain length of the cited n-alkyl sulfates. The present results suggest that the hydrophobic interactions play important roles in stabilizing and preventing the aggregation of the MG state of papain

Choi S. M. and Ma C. Y. (2005) Conformational study of globulin from common buckwheat (*Fagopyrum esculentum* Moench) by Fourier transform infrared spectroscopy and differential scanning calorimetry. *J Agric Food Chem* **53**, 8046-8053.

Abstract: Fourier transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC) were used to study changes in the conformation of globulin from common buckwheat (*Fagopyrum esculentum* Moench) (BWG) under various environmental conditions. The IR spectrum of the native BWG showed several major bands from 1691 to 1636 cm⁻¹ in the amide I' region, and the secondary structure composition was estimated as 34.5% beta-sheets, 20.0% beta-turns, 16.0% alpha-helices, and 14.4% random coils. Highly acidic and alkaline pH conditions induced decreases in beta-sheet and alpha-helical contents, as well as in denaturation temperature (Td) and enthalpy of denaturation (DeltaH), as shown in the DSC thermograms. Addition of chaotropic salts (1.0 M) caused progressive decreases in ordered structures and thermal stability following the lyotropic series of anions. The presence of several protein structure perturbants also led to changes in IR band intensities and DSC thermal stabilities, suggesting protein unfolding. Intermolecular antiparallel beta-sheet (1620 and 1681 cm⁻¹) band intensities started to increase when BWG was heated to 90 degrees C, suggesting the initiation of protein aggregation. Increasing the time of the preheat treatment (at 100 degrees C) caused progressive increases in Td and pronounced decreases in DeltaH, suggesting partial denaturation and reassociation of protein molecules.

Choi S. M., Mine Y., and Ma C. Y. (2006) Characterization of heat-induced aggregates of globulin from common buckwheat (*Fagopyrum esculentum* Moench). *Int J Biol Macromol* **39**, 201-209.

Abstract: Some physicochemical properties and the microstructure of heat-induced aggregates of globulin from common buckwheat (*Fagopyrum esculentum* Moench) (BWG) formed at 100 degrees C in 0.01 M phosphate buffer containing 1.0 M NaCl, pH 7.4 were studied. Differential scanning calorimetric (DSC) analysis shows a re-distribution of native and extensively denatured proteins in the heat-induced aggregates of BWG, particularly in the ISA fraction. Sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS-PAGE) analysis suggests the occurrence of both dissociation and association of molecules and the involvement of intermolecular disulfide linkages during thermal aggregation. Transmission electron microscopy (TEM) reveals that native BWG appeared as uniform compact globules with diameters ranging between 11.7 and 12.5 nm. TEM examination of the buffer-soluble aggregates, fractionated by sucrose density gradient ultracentrifugation, demonstrates the formation of strand-like small aggregates and large compact globular soluble macroaggregates.

Chronakis I. S. (2001) Gelation of edible blue-green algae protein isolate (*Spirulina platensis* Strain Pacifica): thermal transitions, rheological properties, and molecular forces involved. *J Agric Food Chem* **49**, 888-898.

Abstract: Proteins isolated from blue-green algae *Spirulina platensis* strain Pacifica were characterized by visible absorption, differential scanning calorimetry (DSC), viscometry, and dynamic oscillatory rheological measurements. Unique thermal unfolding, denaturation, aggregation, and gelation of the algal protein isolate are presented. DSC analysis showed that thermal transitions occur at about 67 and 109 degrees C at neutral pH. Calcium chloride stabilized the quaternary structure against denaturation and shifted the transitions at higher temperatures. Viscometric studies of *Spirulina* protein isolate as a function of temperature showed that the onset of the viscosity increase is closely related to the dissociation-denaturation process. Lower viscosities were observed for the protein solutions dissolved at pH 9 due to an increased protein solubility. Solutions of *Spirulina* protein isolate form elastic gels during heating to 90 degrees C. Subsequent cooling at ambient temperatures caused a further pronounced increase in the elastic moduli and network elasticity. *Spirulina* protein isolate has good gelling properties with fairly low minimum critical gelling concentrations of about 1.5 and 2.5 wt % in 0.1 M Tris buffer, pH 7, and with 0.02 M CaCl₂ in the same buffer, respectively. It is suggested that mainly the interactions of exposed hydrophobic regions generate the molecular association, initial aggregation, and gelation of the protein

isolate during the thermal treatment. Hydrogen bonds reinforce the network rigidity of the protein on cooling and further stabilize the structure of Spirulina protein gels but alone are not sufficient to form a network structure. Intermolecular sulfhydryl and disulfide bonds were found to play a minor role for the network strength of Spirulina protein gels but affect the elasticity of the structures formed. Both time and temperature at isothermal heat-induced gelation within 40-80 degrees C affect substantially the network formation and the development of elastic modulus of Spirulina protein gels. This is also attributed to the strong temperature dependence of hydrophobic interactions. The aggregation, denaturation, and gelation properties of Spirulina algal protein isolate are likely to be controlled from protein-protein complexes rather than individual protein molecules.

Cobos E. S., Filimonov V. V., Galvez A., Maqueda M., Valdivia E., Martinez J. C., and Mateo P. L. (2001) AS-48: a circular protein with an extremely stable globular structure. *FEBS Lett* **505**, 379-382.

Abstract: The unfolding thermodynamics of the circular enterocin protein AS-48, produced by *Enterococcus faecalis*, has been characterized by differential scanning calorimetry. The native structure of the 70-residue protein is extremely thermally stable. Thus, at pH 2.5 and low ionic strength thermal denaturation occurs under equilibrium at 102 degrees C, while the unfolded state irreversibly aggregates at neutral and alkaline pH. Calorimetric data analysis shows that the specific enthalpy change upon unfolding is unusually small and the heat capacity change is quite normal for a protein of this size, whereas the Gibbs energy change at 25 degrees C is relatively high. At least part of this high stability might be put down to entropic constraints induced by the circular organization of the polypeptide chain.

Collins T., Meuwis M. A., Gerday C., and Feller G. (2003) Activity, stability and flexibility in glycosidases adapted to extreme thermal environments. *J Mol Biol* **328**, 419-428.

Abstract: To elucidate the strategy of low temperature adaptation for a cold-adapted family 8 xylanase, the thermal and chemical stabilities, thermal inactivation, thermodependence of activity and conformational flexibility, as well as the thermodynamic basis of these processes, were compared with those of a thermophilic homolog. Differential scanning calorimetry, fluorescence monitoring of guanidine hydrochloride unfolding and fluorescence quenching were used, among other techniques, to show that the cold-adapted enzyme is characterized by a high activity at low temperatures, a poor stability and a high flexibility. In contrast, the thermophilic enzyme is shown to have a reduced low temperature activity, high stability and a reduced flexibility. These findings agree with the hypothesis that cold-adapted enzymes overcome the quandary imposed by low temperature environments via a global or local increase in the flexibility of their molecular edifice, with this in turn leading to a reduced stability. Analysis of the guanidine hydrochloride unfolding, as well as the thermodynamic parameters of irreversible thermal unfolding and thermal inactivation shows that the driving force for this denaturation and inactivation is a large entropy change while a low enthalpy change is implicated in the low temperature activity. A reduced number of salt-bridges are believed to be responsible for both these effects. Guanidine hydrochloride unfolding studies also indicate that both family 8 enzymes unfold via an intermediate prone to aggregation.

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.

Croy C. H., Bergqvist S., Huxford T., Ghosh G., and Komives E. A. (2004) Biophysical characterization of the free $\text{I}\kappa\text{B}\alpha$ ankyrin repeat domain in solution. *Protein Sci* **13**, 1767-1777.

Abstract: The crystal structure of $\text{I}\kappa\text{B}\alpha$ in complex with the transcription factor, nuclear factor $\kappa\text{-B}$ (NF- κB) shows six ankyrin repeats, which are all ordered. Electron density was not observed for most of the residues within the PEST sequence, although it is required for high-affinity binding. To characterize the folded state of $\text{I}\kappa\text{B}\alpha$ (67-317) when it is not in complex with NF- κB , we have carried out circular dichroism (CD) spectroscopy, 8-anilino-1-naphthalenesulphonic acid (ANS) binding, differential scanning calorimetry, and amide hydrogen/deuterium exchange experiments. The CD spectrum shows the presence of helical structure, consistent with other ankyrin repeat proteins. The large amount of ANS-binding and amide exchange suggest that the protein may have molten globule character. The amide exchange experiments show that the third ankyrin repeat is the most compact, the second and fourth repeats are somewhat less compact, and the first and sixth repeats are solvent exposed. The PEST extension is also highly solvent accessible. $\text{I}\kappa\text{B}\alpha$ unfolds with a T_m of 42 degrees C, and forms a soluble aggregate that

sequesters helical and variable loop parts of the first, fourth, and sixth repeats and the PEST extension. The second and third repeats, which conform most closely to a consensus for stable ankyrin repeats, appear to remain outside of the aggregate. The ramifications of these observations for the biological function of I κ B α are discussed.

D'Amico S., Gerday C., and Feller G. (2001) Structural determinants of cold adaptation and stability in a large protein. *J Biol Chem* **276**, 25791-25796.

Abstract: The heat-labile alpha-amylase from an antarctic bacterium is the largest known protein that unfolds reversibly according to a two-state transition as shown by differential scanning calorimetry. Mutants of this enzyme were produced, carrying additional weak interactions found in thermostable alpha-amylases. It is shown that single amino acid side chain substitutions can significantly modify the melting point T_m , the calorimetric enthalpy $\Delta H(\text{cal})$, the cooperativity and reversibility of unfolding, the thermal inactivation rate constant, and the kinetic parameters $k(\text{cat})$ and K_m . The correlation between thermal inactivation and unfolding reversibility displayed by the mutants also shows that stabilizing interactions increase the frequency of side reactions during refolding, leading to intramolecular mismatches or aggregations typical of large proteins. Although all mutations were located far from the active site, their overall trend is to decrease both $k(\text{cat})$ and K_m by rigidifying the molecule and to protect mutants against thermal inactivation. The effects of these mutations indicate that the cold-adapted alpha-amylase has lost a large number of weak interactions during evolution to reach the required conformational plasticity for catalysis at low temperatures, thereby producing an enzyme close to the lowest stability allowing maintenance of the native conformation.

D'auria S., Rossi M., Barone G., Catanzano F., Del Vecchio P., Graziano G., and Nucci R. (1996) Temperature-induced denaturation of beta-glycosidase from the archaeon *Sulfolobus solfataricus*. *J Biochem (Tokyo)* **120**, 292-300.

Abstract: The beta-glycosidase isolated from the extreme thermophilic archaeon *Sulfolobus solfataricus*, grown at 87 degrees C, is a tetrameric protein with a molecular mass of 240 kDa. This enzyme is barely active at 30 degrees C and has optimal activity, over 95 degrees C, at pH 6.5. Its thermal stability was investigated at pH 10.1 and 10.6 by means of functional studies, circular dichroism and differential scanning calorimetry. There was no evidence of thermal activation of the enzyme and the temperature-induced denaturation was irreversible and not well represented by the two-state transition model. A more complex process occurred, involving the dissociation and unfolding of subunits, and subsequent nonspecific association and/or aggregation. Denaturation temperature was around 85 degrees C, depending on protein concentration. The denaturation enthalpy change was between 7,500 and 9,800 kJ.mol⁻¹, depending on the pH. The collapse of the native structure around 85 degrees C was confirmed by circular dichroism measurements and time-dependent activity studies. Finally, preliminary investigations were performed on the recombinant enzyme expressed in *Escherichia coli*.

D'auria S., Barone R., Rossi M., Nucci R., Barone G., Fessas D., Bertoli E., and Tanfani F. (1997) Effects of temperature and SDS on the structure of beta-glycosidase from the thermophilic archaeon *Sulfolobus solfataricus*. *Biochem J* **323** (Pt 3), 833-840.

Abstract: The effects of temperature and SDS on the three-dimensional organization and secondary structure of beta-glycosidase from the thermophilic archaeon *Sulfolobus solfataricus* were investigated by CD, IR spectroscopy and differential scanning calorimetry. CD spectra in the near UV region showed that the detergent caused a remarkable change in the protein tertiary structure, and far-UV CD analysis revealed only a slight effect on secondary structure. Infrared spectroscopy showed that low concentrations of the detergent (up to 0.02%) induced slight changes in the enzyme secondary structure, whereas high concentrations caused the alpha-helix content to increase at high temperatures and prevented protein aggregation.

Dhalluin C., Ross A., Leuthold L. A., Foser S., Gsell B., Muller F., and Senn H. (2005) Structural and biophysical characterization of the 40 kDa PEG-interferon-alpha2a and its individual positional isomers. *Bioconjug Chem* **16**, 504-517.

Abstract: The human recombinant Interferon-alpha(2a) (IFNalpha(2a)) is a potent drug (Roferon-A) to treat various types of cancer and viral diseases including Hepatitis B/C infections. To improve the pharmacological properties of the drug, a new pegylated form of IFNalpha(2a) was developed

(PEGASYS). This 40 kDa PEG-conjugated IFN α (2a) ((40)PEG-IFN α (2a)) is obtained by the covalent binding of one 40 kDa branched PEG-polymer to a lysine side chain of IFN α (2a). (40)PEG-IFN α (2a) is a mixture of mainly six monopegylated positional isomers modified at K31, K134, K131, K121, K164, and K70, respectively. Here we report the detailed structural and biophysical characterization of (40)PEG-IFN α (2a) and its positional isomers, in comparison with IFN α (2a), using NMR spectroscopy, analytical ultracentrifugation, circular dichroism, fluorescence spectroscopy, and differential scanning calorimetry. Our results show that the three-dimensional structure of IFN α (2a) is not modified by the presence of the polymer in all positional isomers constituting (40)PEG-IFN α (2a). Regardless of where the PEG-polymer is attached, it adopts a very mobile and flexible random coil conformation, producing a shield for the protein without a permanent coverage of the protein surface. Hydrodynamic data indicate that the protein-attached PEG has a slightly more compact random-coil structure than the free PEG-polymer. Our results also provide evidence of significant structural and physicochemical advantages conferred by the pegylation: increase of the effective hydrodynamic volume and modification of the molecular shape, higher temperature stability, and reduced tendency for aggregation. These results are of tremendous pharmacological interest and benefit as was clinically shown with PEGASYS. This study constitutes a new standard for the characterization of pegylated proteins and enables an important step toward the understanding on a molecular level of the binding of (40)PEG-IFN α (2a) and its positional isomers to its cellular receptors.

Duranti M., Sessa F., Scarafoni A., Bellini T., and Dallochio F. (2000) Thermal stabilities of lupin seed conglutin gamma protomers and tetramers. *J Agric Food Chem* **48**, 1118-1123.

Abstract: Various experimental approaches have been used in this work to assess the thermal stabilities of lupin seed conglutin gamma at two pH values, 4.5 and 7.5, at which the protein exists as a protomer and a tetramer, respectively. The patterns of thermal unfolding at the two pH values differed significantly; the tetramer aggregated and became insoluble, whereas the protomer was still soluble after thermal treatment. Also, the midpoint transition temperatures were dramatically different, being 60.3 and 75.1 degrees C for the protomer and tetramer, respectively. The behavior of conglutin gamma at neutral pH was also affected by disulfide formation/interchange, in that some unfolded protein molecules became covalently stabilized. More detailed analyses by differential scanning calorimetry and indirect fluorescence measurements, using 8-anilino-1-naphthalenesulfonic acid as a probe, confirmed the remarkable differences observed in the thermal stabilities of the two protein forms and allowed models for their unfolding patterns to be drawn.

Duy C., and Fitter J. (2005) Thermostability of irreversible unfolding alpha-amylases analyzed by unfolding kinetics. *J Biol Chem.* **280**, 37360-5.

Abstract: For most multidomain proteins the thermal unfolding transitions are accompanied by an irreversible step, often related to aggregation at elevated temperatures. As a consequence the analysis of thermostabilities in terms of equilibrium thermodynamics is not applicable, at least not if the irreversible process is fast with respect the structural unfolding transition. In a comparative study we investigated aggregation effects and unfolding kinetics for five homologous alpha-amylases, all from mesophilic sources but with rather different thermostabilities. The results indicate that for all enzymes the irreversible process is fast and the precedent unfolding transition is the rate-limiting step. In this case the kinetic barrier toward unfolding, as measured by unfolding rates as function of temperature, is the key feature in thermostability. The investigated enzymes exhibit activation energies (E(a)) between 208 and 364 kJmol(-1) and pronounced differences in the corresponding unfolding rates. The most thermostable alpha-amylase from *Bacillus licheniformis* (apparent transition temperature, T(1/2) approximately 100 degrees C) shows an unfolding rate which is four orders of magnitude smaller as compared with the alpha-amylase from pig pancreas (T(1/2) approximately 65 degrees C). Even with respect to two other alpha-amylases from *Bacillus* species (T(1/2) approximately 86 degrees C) the difference in unfolding rates is still two orders of magnitude.

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

Abstract: The interaction of left- and right-handed polylysine chains (poly(D-lysine) and poly(L-lysine)) results in a dramatic increase in the propensity to form aggregated beta-sheet structure (and amyloid-like fibrils), which is reflected by an approximately 15 degrees C decrease of temperature of the alpha-helix-to-

beta-sheet transition. While a relative volume expansion of 13-19 mL x mol⁻¹ accompanies the alpha-to-beta-transition in a single enantiomer, this does not hold true for the mixture, which, along with substantially more negative heat capacity changes, points to a lower solvent-entropy cost of the transition as the possible thermodynamic driving force of the diastereomeric aggregation. The underlying solvational mechanism may be one of the decisive factors responsible for the spontaneous protein aggregation in vivo and, as such, may shed new light on the molecular basis of amyloid-associated diseases.

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

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

Ejima D., Tsumoto K., Fukada H., Yumioka R., Nagase K., Arakawa T. and Philo J. S. (2007) Effects of acid exposure on the conformation, stability, and aggregation of monoclonal antibodies. *Proteins* **66**, 954-962.

Abstract: Exposure of antibodies to low pH is often unavoidable for purification and viral clearance. The conformation and stability of two humanized monoclonal antibodies (hIgG4-A and -B) directed against different antigens and a mouse monoclonal antibody (mIgG1) in 0.1M citrate at acidic pH were studied using circular dichroism (CD), differential scanning calorimetry (DSC), and sedimentation velocity. Near- and far-UV CD spectra showed that exposure of these antibodies to pH 2.7-3.9 induced only limited conformational changes, although the changes were greater at the lower pH. However, the acid conformation is far from unfolded or so-called molten globule structure. Incubation of hIgG4-A at pH 2.7 and 3.5 at 4 degrees C over the course of 24 h caused little change in the near-UV CD spectra, indicating that the acid conformation is stable. Sedimentation velocity showed that the hIgG4-A is largely monomeric at pH 2.7 and 3.5 as well as at pH 6.0. No time-dependent changes in sedimentation profile occurred upon incubation at these low pHs, consistent with the conformational stability observed by CD. The sedimentation coefficient of the monomer at pH 2.7 or 3.5 again suggested that no gross conformational changes occur at these pHs. DSC analysis of the antibodies showed thermal unfolding at pH 2.7-3.9 as well as at pH 6.0, but with decreased melting temperatures at the lower pH. These results are consistent with the view that the antibodies undergo limited conformational change, and that incubation at 4 degrees C at low pH results in no time-dependent conformational changes. Titration of hIgG4-A from pH 3.5 to 6.0 resulted in recovery of native monomeric proteins whose CD and DSC profiles resembled those of the original sample. However, titration from pH 2.7 resulted in lower recovery of monomeric antibody, indicating that the greater conformational changes observed at this pH cannot be fully reversed to the native structure by a simple pH titration,

Espinosa L., Schebor C., Nudelman N. S., and Chirife J. (2004) Stability of enzymes and proteins in dried glassy systems: effect of simulated sunlight conditions. *Biotechnol Prog* **20**, 1220-1224.

Abstract: The purpose of the present work was to study the effects of simulated sunlight conditions on enzyme inactivation and structural damage in dehydrated glassy systems. Freeze-dried samples containing different enzymes (lactase, invertase, lysozyme and amyloglucosidase) were exposed to light using a medium-pressure metal halide HPA 400 W lamp. After 1 h of light exposure, the samples showed a

significant reduction (more than 50%) in the denaturation peak area as analyzed by DSC, and this could be attributed to protein denaturation. For most of the pure enzymes, the loss of enzymic activity after 1 h of light exposure was around 50%. In the case of enzymes included in anhydrous model systems (trehalose, raffinose, maltodextrin, and dextran), the remaining activity also decreased dramatically during the light treatment. We showed that the light exposure in dehydrated systems generated both the loss of enzymic activity and structural changes such as denaturation (observed by DSC) and protein fragmentation and aggregation (observed by electrophoresis). Overall, we can conclude that a short exposure to the light produces dramatic changes in the enzymic activity in dehydrated systems with or without protective matrices.

Faria T. Q., Knapp S., Ladenstein R., Macanita A. L., and Santos H. (2003) Protein stabilisation by compatible solutes: effect of mannosylglycerate on unfolding thermodynamics and activity of ribonuclease A. *Chembiochem* **4**, 734-741.

Abstract: Differential scanning calorimetry, optical spectroscopy, and activity measurements were used to investigate the effect of mannosylglycerate, a negatively charged osmolyte widely distributed among thermophilic and hyperthermophilic archaea and bacteria, on the thermal unfolding of ribonuclease A (RNase A). For comparison, assays in the presence of trehalose, a canonical solute in mesophiles, and potassium chloride were also carried out. A thermodynamic analysis was performed by using differential scanning calorimetry data. The changes in the heat capacity for unfolding were similar for the different solutes examined. Mannosylglycerate was an efficient thermostabiliser of RNase A and induced an increase of 6 degrees C mole⁻¹ in the melting temperature. Moreover, the performance of mannosylglycerate as a stabiliser depended on the net charge of the molecule, with the maximal effect being observed at pH values above 4.5. Analysis of the enthalpic and entropic contributions to unfolding, derived from calorimetric data, revealed that the stabilisation rendered by mannosylglycerate is primarily achieved through a decrease in the unfolding entropy. Also, the number of protons taken up by RNase A upon denaturation in the presence of mannosylglycerate was considerably higher than with other solutes, a result consistent with a more rigid structure of the native protein. Mannosylglycerate (potassium salt) inhibited the activity of RNase A, albeit to a smaller extent than KCl, and acted as an efficient suppressor of aggregation of the denatured protein, thereby having a remarkable beneficial effect on the inactivation of RNase A upon thermal denaturation. The results are discussed in view of the physiological role of this charged compatible solute.

Farruggia B. and Pico G. A. (1999) Thermodynamic features of the chemical and thermal denaturations of human serum albumin. *Int J Biol Macromol* **26**, 317-323.

Abstract: The unfolding process of human serum albumin between pH 5.4 and 9.9 was studied by chemical and thermal denaturations. The experimental results showed that there is no correlation between the stability of albumin at different pH values determined by both methods. The free energy change of unfolding versus concentration of guanidine showed a close dependence on the pH, suggesting that the variation of the electrical charge of albumin influences the final state of the unfolded form of the protein. Spectroscopic techniques, such as native fluorescence of the protein and circular dichroism, demonstrated that the unfolded state of the protein obtained from both methods possesses a different helical content. The solvophobic effect and the entropy of the chains have no influence on the final unfolding state when the protein is unfolded by thermal treatment, while, when the protein is unfolded by chemical denaturants, both effects depend on the medium pH. The results indicate that guanidine and urea interact with albumin by electrostatic forces, yielding a randomly coiled conformation in its unfolded state, while thermal denaturation produces a molten globule state and the aggregation of the protein; therefore, both methods yield different structurally unfolded states of the albumin.

Fedunova D. and Antalik M. (2006) Prevention of thermal induced aggregation of cytochrome c at isoelectric pH values by polyanions. *Biotechnol Bioeng* **93**, 485-493.

Abstract: Differential scanning calorimetry, viscometry, optical and CD spectroscopy were used to characterize the influence of two polyanions, poly(vinylsulfate) (PVS), and poly(4-styrene-sulfonate) (PSS) on thermal transition reversibility of ferricytochrome c at or near isoelectric pH. In these conditions, both PVS and PSS enhance the thermal transition reversibility of cytochrome c by preventing the aggregation of denatured protein molecules. Data indicate that the polyanions are in complex with cytochrome c that is stabilized by synergistic effect of Coulombic and non-Coulombic interactions.

Fedunova D. and Antalík M. (1998) Studies on interactions between metmyoglobin and heparin. *Gen Physiol Biophys* **17**, 117-131.

Abstract: The complex formation between metmyoglobin and heparin was investigated by absorbance and fluorescence spectroscopy as well as differential scanning microcalorimetry. In acidic pH region, three distinct complexes detected by absorbance measurements are formed depending on pH and time of equilibration. The kinetics of the conformational transition of metmyoglobin-heparin complex equilibrated at neutral pH observed after pH change to acidic region comprises two steps. During the first step, characterized by rapid changes of the absorption spectra (approximately 5 minutes) as well as fluorescence intensities, reversible transition with $pK = 6.5 \pm 0.1$ occurs and the first type of the complex forms. Below pH 6.2 the transition with $pK = 5.7 \pm 0.1$ is observed and the second type of the complex is formed. During the second slow step, the third type of the complex formed after 30 minutes of equilibration is characterized by a spectrum corresponding to low-spin form without protein axial ligand bound. At neutral pH and 25 degrees C, the interaction between metMb and heparin only slightly alters absorption and fluorescence spectra. On the other hand, the formation of metMb-heparin complex is established from the decrease of the transition temperature from 80.4 ± 0.5 degrees C to 74.7 ± 0.5 degrees C. Moreover, the binding of heparin prevents the aggregation of the protein at isoelectric point resulting in a considerable increase in the reversibility of thermal denaturation.

Fedurkina N. V., Belousova L. V., Mitskevich L. G., Zhou H. M., Chang Z., and Kurganov B. I. (2006) Change in kinetic regime of protein aggregation with temperature increase. Thermal aggregation of rabbit muscle creatine kinase. *Biochemistry (Mosc)* **71**, 325-331.

Abstract: Creatine kinase thermal aggregation kinetics has been studied in 30 mM Hepes-NaOH buffer, pH 8.0, at two temperatures: 50.6 and 60 degrees C. Aggregation kinetics was analyzed by measuring the growth of apparent absorption (A) at 400 nm. It was found that the limiting value of apparent absorption (A(lim)) is proportional to protein concentration at both temperatures. The first order rate constant (k(I)) does not depend on protein concentration in the range 0.05-0.2 mg/ml at temperature 50.6 degrees C, but at temperature 60 degrees C it increases with the growth of protein concentration in the range 0.1-0.4 mg/ml. Kinetic curves, shown in coordinates $\{A/A(lim); t\}$, in experiments at 50.6 degrees C fuse to a common curve, which coincides with the theoretical curve of creatine kinase denaturation calculated using the denaturation rate constant determined from differential scanning calorimetry. At temperature 60 degrees C, half-transformation time $t(1/2) = \ln 2/k(I)$ decreases when protein concentration grows. We conclude that when temperature increased from 50.6 to 60 degrees C, change in the kinetic regime of thermal creatine kinase aggregation took place: at 50.6 degrees C aggregation rate is limited by the stage of protein molecule denaturation, but at 60 degrees C it is limited by the stage of protein aggregate growth, which proceeds as a reaction of pseudo-first order. Small heat shock protein Hsp 16.3 Mycobacterium tuberculosis suppresses the creatine kinase aggregation.

Fernandez A. M., Villegas V., Martínez J. C., van Nuland N. A., Conejero-Lara F., Aviles F. X., Serrano L., Filimonov V. V., and Mateo P. L. (2000) Thermodynamic analysis of helix-engineered forms of the activation domain of human procarboxypeptidase A2. *Eur J Biochem* **267**, 5891-5899.

Abstract: Thermodynamic characterization of the activation domain of human procarboxypeptidase A2, ADA2h, and its helix-engineered mutants was carried out by differential scanning calorimetry. The mutants were engineered by changing residues in the exposed face of the two alpha helices in order to increase their stability. At neutral and alkaline pH the three mutants, alpha-helix 1 (M1), alpha-helix 2 (M2) and alpha-helix 1 and alpha-helix 2 (DM), were more stable than the wild-type domain, in the order DM, M2, M1 and wild-type. Under these conditions the CD and NMR spectra of all the variants are very similar, indicating that this increase in stability is not the result of gross structural changes. Calorimetric analysis shows that the stabilizing effect of mutating the water-exposed surfaces of the helices seems to be mainly entropic, because the mutations do not change the enthalpy or the increase in heat capacity of denaturation. The unfolding behavior of all variants changes under acidic conditions: whereas wild-type and M1 have a strong tendency to aggregate, giving rise to a beta conformation upon unfolding, M2 and DM unfold reversibly, M2 being more stable than DM. CD and NMR experiments at pH 3.0 suggest that a region involving residues of the second and third beta strands as well as part of alpha-helix 1 changes its conformation. It seems that the enhanced stability of the altered conformation of M2 and DM reduces the aggregation tendency of ADA2h at acidic pH.

Fukumura M., Tanaka A., Sakka K., and Ohmiya K. (1995) Process of thermal denaturation of xylanase (XynB) from *Clostridium stercorarium* F-9. *Biosci Biotechnol Biochem* **59**, 47-50.

Abstract: The thermal denaturation process of *Clostridium stercorarium* F-9 xylanase (XynB) was studied by monitoring remaining activity and recovered activity of the enzyme. At pH 5.5, aggregation occurred rapidly after the thermal denaturation initiated. The aggregated protein could be dissolved in 8 M urea solution, and the enzyme activity was recovered by diluting the urea. The extent of the recovered activity was gradually decreased with two phases as the reaction time of the thermal denaturation became longer. These results suggested the thermal denaturation process to be as follows: [formula: see text] where N is the native state of the enzyme; D1 is the denatured state of the enzyme that is formed rapidly after the reaction started and can be renatured by the urea treatment, and D2 and D3 are the denatured states of the enzyme that cannot be renatured even by the urea treatment. The rate constants were $k_1 > 9.2$, $k_2 = 0.33$, and $k_3 = 0.57$, and $k_3 = 0.13$ (in min⁻¹ unit).

Furukawa Y. and O'Halloran T. V. (2005) Amyotrophic lateral sclerosis mutations have the greatest destabilizing effect on the apo- and reduced form of SOD1, leading to unfolding and oxidative aggregation. *J Biol Chem* **280**, 17266-17274.

Abstract: Mutant forms of Cu,Zn-superoxide dismutase (SOD1) that cause familial amyotrophic lateral sclerosis (ALS) exhibit toxicity that promotes the death of motor neurons. Proposals for the toxic properties typically involve aberrant catalytic activities or protein aggregation. The striking thermodynamic stability of mature forms of the ALS mutant SOD1 ($T_m > 70$ degrees C) is not typical of protein aggregation models that involve unfolding. Over 44 states of the polypeptide are possible, depending upon metal occupancy, disulfide status, and oligomeric state; however, it is not clear which forms might be responsible for toxicity. Recently the intramolecular disulfide has been shown to be required for SOD1 activity, leading us to examine these states of several disease-causing SOD1 mutants. We find that ALS mutations have the greatest effect on the most immature form of SOD1, destabilizing the metal-free and disulfide-reduced polypeptide to the point that it is unfolded at physiological temperatures ($T_m < 37$ degrees C). We also find that immature states of ALS mutant (but not wild type) proteins readily form oligomers at physiological concentrations. Furthermore, these oligomers are more susceptible to mild oxidative stress, which promotes incorrect disulfide cross-links between conserved cysteines and drives aggregation. Thus it is the earliest disulfide-reduced polypeptides in the SOD1 assembly pathway that are most destabilized with respect to unfolding and oxidative aggregation by ALS-causing mutations.

Garcia-Garcia J., Gomez-Fernandez J. C., and Corbalan-Garcia S. (2001) Structural characterization of the C2 domain of novel protein kinase Cepsilon. *Eur J Biochem* **268**, 1107-1117.

Abstract: Infrared spectroscopy (IR) and differential scanning calorimetry (DSC) were used to study the biophysical properties of the PKCepsilon-C2 domain, a C2 domain that possess special characteristics as it binds to acidic phospholipids in a Ca²⁺-independent manner and no structural information about it is available to date. When the secondary structure was determined by IR spectroscopy in H₂O and D₂O buffers, beta sheet was seen to be the major structural component. Spectroscopic studies of the thermal denaturation in D₂O showed a broadening in the amide I' band starting at 45 degrees C. Curve fitting analysis of the spectra demonstrated that two components appear upon thermal denaturation, one at 1623 cm⁻¹ which was assigned to aggregation and a second one at 1645 cm⁻¹, which was assigned to unordered or open loop structures. A lipid binding assay has demonstrated that PKCepsilon-C2 domain has preferential affinity for PIP₂ although it exhibits maximal binding activity for phosphatidic acid when 100 mol% of this negatively charged phospholipid was used. Thus, phosphatidic acid containing vesicles were used to characterize the effect of lipid binding on the secondary structure and thermal stability. These experiments showed that the secondary structure did not change upon lipid binding and the thermal stability was very high with no significant changes occurring in the secondary structure after heating. DSC experiments demonstrated that when the C2-protein was scanned alone, it showed a T_m of 49 degrees C and a calorimetric denaturation enthalpy of 144.318 kJ x mol⁻¹. However, when phosphatidic acid vesicles were included in the mixture, the transition disappeared and further IR experiments demonstrated that the protein structure was not modified under these conditions.

Gicquaud C. R., Aubin P. H., Heppell B., and St Gelais F. (2005) F-actin has a very high calorimetric unfolding enthalpy. *Biochem Biophys Res Commun* **334**, 145-149.

Abstract: The thermal unfolding of F-actin was studied using differential scanning calorimetry. Heat

denatures F-actin in two steps. The first is endothermic and corresponds to the unfolding of the peptide chain, while the second is exothermic and is due to the aggregation of the unfolded molecules. The aspect of the thermogram is influenced by the concentration of the protein. For concentrations around 1mg/ml, the steps are superimposed, while the two steps are separated at very low concentrations. It thus becomes possible to estimate the calorimetric enthalpy for the unfolding step. The enthalpy of unfolding is 64 MJ/mol, or 1400 J/g. This value is considerably higher than those mentioned in the literature for the denaturation of actin and other proteins, which are in the range of 25-30 J/g. The large amount of energy required to unfold the molecule of F-actin could be an adaptation of its role as a protein that transmits forces, and consequently must be very resistant to mechanical constraints.

Gomez-Orellana I., Variano B., Miura-Fraboni J., Milstein S., and Paton D. R. (1998) Thermodynamic characterization of an intermediate state of human growth hormone. *Protein Sci* **7**, 1352-1358.

Abstract: The thermal denaturation of recombinant human growth hormone (rhGH) was studied by differential scanning calorimetry and circular dichroism spectroscopy (CD). The thermal unfolding is reversible only below pH 3.5, and under these conditions a single two-state transition was observed between 0 and 100 degrees C. The magnitudes of the ΔH and ΔC_p of this transition indicate that it corresponds to a partial unfolding of rhGH. This is also supported by CD data, which show that significant secondary structure remains after the unfolding. Above pH 3.5 the thermal denaturation is irreversible due to the aggregation of rhGH upon unfolding. This aggregation is prevented in aqueous solutions of alcohols such as n-propanol, 2-propanol, or 1,2-propanediol (propylene glycol), which suggests that the self-association of rhGH is caused by hydrophobic interactions. In addition, it was found that the native state of rhGH is stable in relatively high concentrations of propylene glycol (up to 45% v/v at pH 7-8 or 30% at pH 3) and that under these conditions the thermal unfolding is cooperative and corresponds to a transition from the native state to a partially folded state, as observed at acidic pH in the absence of alcohols. In higher concentrations of propylene glycol, the tertiary structure of rhGH is disrupted and the cooperativity of the unfolding decreases. Moreover, the CD and DSC data indicate that a partially folded intermediate with essentially native secondary structure and disordered tertiary structure becomes significantly populated in 70-80% propylene glycol.

Goodman J. L., Petersson E. J., Daniels D. S., Qiu J. X. and Schepartz A. (2007) Biophysical and structural characterization of a robust octameric beta-peptide bundle. *J Am. Chem Soc.* **129**, 14746-14751.

Abstract: Proteins composed of alpha-amino acids are essential components of the machinery required for life. Stanley Miller's renowned electric discharge experiment provided evidence that an environment of methane, ammonia, water, and hydrogen was sufficient to produce alpha-amino acids. This reaction also generated other potential protein building blocks such as the beta-amino acid beta-glycine (also known as beta-alanine); however, the potential of these species to form complex ordered structures that support functional roles has not been widely investigated. In this report we apply a variety of biophysical techniques, including circular dichroism, differential scanning calorimetry, analytical ultracentrifugation, NMR and X-ray crystallography, to characterize the oligomerization of two 12-mer beta3-peptides, Acid-1Y and Acid-1Y*. Like the previously reported beta3-peptide Zwit-1F, Acid-1Y and Acid-1Y* fold spontaneously into discrete, octameric quaternary structures that we refer to as beta-peptide bundles. Surprisingly, the Acid-1Y octamer is more stable than the analogous Zwit-1F octamer, in terms of both its thermodynamics and kinetics of unfolding. The structure of Acid-1Y, reported here to 2.3 Å resolution, provides intriguing hypotheses for the increase in stability. To summarize, in this work we provide additional evidence that nonnatural beta-peptide oligomers can assemble into cooperatively folded structures with potential application in enzyme design, and as medical tools and nanomaterials. Furthermore, these studies suggest that nature's selection of alpha-amino acid precursors was not based solely on their ability to assemble into stable oligomeric structures,

Goyal M. K., Roy I., Banerjee U. C., Sharma V. K. and Bansal A. K. (2008) Role of benzyl alcohol in the prevention of heat-induced aggregation and inactivation of hen egg white lysozyme. *Eur J Pharm. Biopharm (epublication)*

Abstract: The aim of the study was to investigate the stability of a model protein, lysozyme, in the presence of the commonly used preservative benzyl alcohol. Techniques including lytic assay, size exclusion chromatography, circular dichroism, differential scanning calorimetry, native polyacrylamide gel electrophoresis and dynamic light scattering were used to study the overall stability of lysozyme in the

presence of benzyl alcohol. The stability of lysozyme against thermal stress was higher in the presence of benzyl alcohol. In the presence of 0.5%, 0.9% and 2% v/v benzyl alcohol, the enzyme showed 33%, 42% and 75% residual activity, respectively, when exposed to 75 degrees C for 2h, as compared to the 22% activity of control sample. A gradual increase in the size of aggregates was observed for the control sample relative to the samples containing benzyl alcohol, as a result of loss of monomer concentration. The effect was found to be concentration-dependent with 2% benzyl alcohol showing maximum prevention of heat-induced unfolding and aggregation. This effect is remarkable since the thermal transition temperature of the enzyme decreases in the presence of benzyl alcohol. Benzyl alcohol favours the thermal denaturation of lysozyme but stabilizes the lysozyme against the heat-induced aggregation

Grillo A. O., Edwards K. L., Kashi R. S., Shipley K. M., Hu L., Besman M. J., and Middaugh C. R. (2001) Conformational origin of the aggregation of recombinant human factor VIII. *Biochemistry* **40**, 586-595.

Abstract: Aggregation of proteins is a major problem in their use as drugs and is also involved in a variety of pathological diseases. In this study, biophysical techniques were employed to investigate aggregate formation in the pharmaceutically important protein, recombinant human factor VIII (rhFVIII). Recombinant human factor VIII incubated in solution at 37 degrees C formed soluble aggregates as detected by molecular sieve chromatography and dynamic light scattering. This resulted in a corresponding loss of biological activity. Fluorescence and CD spectra of the thermally stressed rhFVIII samples did not, however, suggest significant differences in protein conformation. To identify conformational changes in rhFVIII that may be involved in rhFVIII aggregation, temperature and solutes were used to perturb the native structure of rhFVIII. Far-UV CD and FTIR studies of rhFVIII as a function of temperature revealed conformational changes corresponding to an increase in intermolecular beta-sheet content beginning at approximately 45 degrees C with significant aggregation observed above 60 degrees C. Fluorescence and DSC studies of rhFVIII also indicated conformational changes initiating between 45 and 50 degrees C. An increase in the exposure of hydrophobic surfaces was observed beginning at approximately 40 degrees C, as monitored by increased binding of the fluorescent probe, bis-anilino-naphthalene sulfonic acid (bis-ANS). Perturbation by various solutes produced several transitions prior to extensive unfolding of rhFVIII. In all cases, a common transition, characterized by an increase in the wavelength of the fluorescence emission maximum of rhFVIII from approximately 330 to 335 nm, was observed during thermal and solute perturbation of factor VIII. Moreover, this transition was correlated with an increased association of factor VIII upon incubation at 37 degrees C in the presence of various solutes. These results suggest that association of rhFVIII in solution was initiated by a small transition in the tertiary structure of the protein which produced a nucleating species that led to the formation of inactive soluble aggregates.

Harn N., Allan C., Oliver C. and Middaugh C. R. (2007) Highly concentrated monoclonal antibody solutions: direct analysis of physical structure and thermal stability. *J Pharm. Sci* **96**, 532-546.

Abstract: Virtually all current analytical methods employed in the development of highly concentrated monoclonal antibody (MAb) formulations require dilution of the sample before acquiring data. Thus, there is an unmet need for methods to study proteins directly at high concentration, since extrapolation of stability indicating parameters obtained from dilute studies may not be representative of the high concentration solution. Only slight or no modifications of biophysical methods including fluorescence, UV absorbance, circular dichroism, and FTIR (ATR and transmittance) spectroscopies as well as differential scanning calorimetry (DSC) are described here that permit the direct study of immunoglobulins (and other proteins) at high concentrations. Although FTIR spectra show differences that are dependent upon sampling geometry, other spectroscopic data from two different recombinant MAbs suggests that structure of each antibody exists in a physically similar state in the concentration range of 0.1-190 mg/mL in 40 mM pH 6 citrate-phosphate buffer. Upon thermally stressing these proteins, spectroscopic techniques that probe tertiary structure demonstrate a decrease in the apparent thermal melting temperature of approximately 5-20 degrees C of both proteins with increasing concentration. In contrast, DSC thermograms and CD thermal experiments suggest a minor degree of stabilization (approximately 2 degrees C) for both antibodies although protein association could be responsible for these observations. Empirical phase diagrams produced from spectroscopic data also suggest (1) the existence of similar structural states at low temperatures independent of concentration and (2) a decrease in the temperature at which phase changes are observed with increasing concentration. The decrease in structural stability observed in these studies is probably the result of aggregation or self-association of the recombinant MAbs upon heating in crowded solutions and not due to a decrease in the intrinsic structural stability of the MAbs.

Hashim S. O., Kaul R. H., Andersson M., Mula F. J., and Mattiasson B. (2005) Differential scanning calorimetric studies of a *Bacillus halodurans* alpha-amylase. *Biochim Biophys Acta* **1723**, 184-191.

Abstract: The thermal unfolding of Amy 34, a recombinant alpha-amylase from *Bacillus halodurans*, has been investigated using differential scanning calorimetry (DSC). The denaturation of Amy 34 involves irreversible processes with an apparent denaturation temperature ($T(m)$) of 70.8 degrees C at pH 9.0, with four transitions, as determined using multiple Gaussian curves. The $T(m)$ increased by 5 degrees C in the presence of 100-fold molar excess of $CaCl_2$ while the aggregation of Amy 34 was observed in the presence of 1000-fold molar excess of $CaCl_2$. Increase in the calcium ion concentration from 1- to 5-fold molar excess resulted in an increase in calorimetric enthalpy ($\Delta H(cal)$), however, at higher concentrations of $CaCl_2$ (up to 100-fold), $\Delta H(cal)$ was found to decrease, accompanied by a decrease in entropy change (ΔS), while the $T(m)$ steadily increased. The presence of 100-fold excess of metal chelator, EDTA, resulted in a decrease in $T(m)$ by 10.4 degrees C. $T(m)$ was also decreased to 61.1 degrees C and 65.9 degrees C at pH 6.0 and pH 11.0, respectively.

He H.W., Zhang J., Zhou H.M., and Yan Y.B. (2005) Conformational change in the C-terminal domain is responsible for the initiation of creatine kinase thermal aggregation. *Biophys J.* **89**, 2650-8.

Abstract: Protein conformational changes may be associated with particular properties such as its function, transportation, assembly, tendency to aggregate, and potential cytotoxicity. In this research, the conformational change that is responsible for the fast destabilization and aggregation of rabbit muscle creatine kinase (EC 2.7.3.2) induced by heat was studied by intrinsic fluorescence and infrared spectroscopy. A pretransitional change of the tryptophan microenvironments was found from the intrinsic fluorescence spectra. A further analysis of the infrared spectra using quantitative second-derivative and two-dimensional correlation analysis indicated that the changes of the beta-sheet structures in the C-terminal domain and the loops occurred before the formation of intermolecular cross-beta-sheet structures and the unfolding of alpha-helices. These results suggested that the pretransitional conformational changes in the active site and the C-terminal domain might result in the modification of the domain-domain interactions and the formation of an inactive dimeric form that was prone to aggregate. Our results highlighted the fact that some minor conformational changes, which were usually negligible or undetectable by normal methods, might play a crucial role in protein stability and aggregation. Our results also suggested that the changes in domain-domain interactions, but not the dissociation of the dimer, might play a crucial role in the thermal denaturation and aggregation of this dimeric two-domain protein.

Hess J. M. and Kelly R. M. (1999) Influence of polymolecular events on inactivation behavior of xylose isomerase from *Thermotoga neapolitana* 5068. *Biotechnol Bioeng* **62**, 509-517.

Abstract: The inactivation behavior of the xylose isomerase from *Thermotoga neapolitana* (TN5068 XI) was examined for both the soluble and immobilized enzyme. Polymolecular events were involved in the deactivation of the soluble enzyme. Inactivation was biphasic at 95 degrees C, pH 7.0 and 7.9, the second phase was concentration-dependent. The enzyme was most stable at low enzyme concentrations, however, the second phase of inactivation was 3- to 30-fold slower than the initial phase. Both phases of inactivation were more rapid at pH 7.9, relative to 7.0. Differential scanning calorimetry of the TN5068 XI revealed two distinct thermal transitions at 99 degrees and 109 degrees C. The relative magnitude of the second transition was dramatically reduced at pH 7.9 relative to pH 7.0. Approximately 24% and 11% activity were recoverable after the first transition at pH 7.0 and 7.9, respectively. When the TN5068 XI was immobilized by covalent attachment to glass beads, inactivation was monophasic with a rate corresponding to the initial phase of inactivation for the soluble enzyme. The immobilized enzyme inactivation rate corresponded closely to the rate of ammonia release, presumably from deamidation of labile asparagine and/or glutamine residues. A second, slower inactivation phase suggests the presence of an unfolding intermediate, which was not observed for the immobilized enzyme. The concentration dependence of the second phase of inactivation suggests that polymolecular events were involved. Formation of a reversible polymolecular aggregate capable of protecting the soluble enzyme from irreversible deactivation appears to be responsible for the second phase of inactivation seen for the soluble enzyme. Whether this characteristic is common to other hyperthermophilic enzymes remains to be seen.

Hoiberg-Nielsen R., Fuglsang C. C., Arleth L., and Westh P. (2006) Interrelationships of glycosylation and aggregation kinetics for *Peniophora lycii* phytase. *Biochemistry* **45**, 5057-5066.

Abstract: The kinetics of thermally induced aggregation of the glycoprotein *Peniophora lycii* phytase (Phy) and a deglycosylated form (dgPhy) was studied by dynamic (DLS) and static (SLS) light scattering. This provided a detailed insight into the time course of the formation of small aggregates (approximately 10-100 molecules) of the enzyme. The thermodynamic stability of the two forms was also investigated using scanning calorimetry (DSC). It was found that the glycans strongly promoted kinetic stability (i.e., reduced the rate of irreversible denaturation) while leaving the equilibrium denaturation temperature, $T(d)$, defined by DSC, largely unaltered. At pH 4.5-5.0, for example, dgPhy aggregated approximately 200 times faster than Phy, even though the difference in $T(d)$ was only 1-3 degrees C. To elucidate the mechanism by which the glycans promote kinetic stability, we measured the effect of ionic strength and temperature on the aggregation rate. Also, the second virial coefficients ($B(22)$) for the two forms were measured by SLS. These results showed that the aggregation rate of Phy scaled with the concentration of thermally denatured protein. This suggested first-order kinetics with respect to the concentration of the thermally denatured state. A similar but less pronounced correlation was found for dgPhy, and it was suggested that while the aggregation process for the deglycosylated form is dominated by denatured protein, it also involves a smaller contribution from associating molecules in the native state. The measurements of $B(22)$ revealed that dgPhy had slightly higher values than Phy. This suggests that dgPhy interacts more favorably with the buffer than Phy and hence rules out strong hydration of the glycans as the origin of their effect on the kinetic stability. On the basis of this and the effects of pH and ionic strength, we suggest that the inhibition of aggregation is more likely to depend on steric hindrance of the glycans in the aggregated form of the protein.

Hsu E., Osslund T., Nybo R., Chen B. L., Kenney W. C., Morris C. F., Arakawa T., and Narhi L. O. (2006) Enhanced stability of recombinant keratinocyte growth factor by mutagenesis. *Protein Eng Des Sel* **19**, 147-153.

Abstract: Native sequence keratinocyte growth factor (KGF) is fairly unstable, as manifested by the loss of the monomeric native protein accompanied by the accumulation of aggregated species during storage at moderate temperatures. Several different types of analogs were generated and the storage stability of the protein assessed. In the first type of analog one or more of the five cysteinyl residues in KGF were replaced; in the second class the N-terminal residues that included the first disulfide bond were deleted. Both of these types of analogs involved removal of the disulfide bond between cysteines 1 and 15. The third group involved mutating one of the basic amino acids located in a cluster of positive charges (involved in heparin binding) around Arg144 to a neutral or acidic amino acid residue. Among the cysteine replacement analogs, the double mutation of Cys1 and 15 to Ser resulted in significantly increased stability without compromising the mitogenic activity, while Cys to Ser mutations at other positions were either destabilizing or had no effect. Deletion of the 15, 23 or 27 N-terminal amino acid residues also increased the stability of the protein. The activity of the analogs was not affected by the deletion of 15 or 23 amino acids, but it was significantly decreased upon removal of the 27 N-terminal amino acid residues. Much greater stability was achieved by mutation of the basic amino acids, especially Arg144, to Glu or Gln, but this increase in stability was accompanied by large decrease in activity. The analog with the 23 N-terminal amino acid residues deleted represents one of the best compromises between increased stability and retention of activity.

Ihnat P. M., Vellekamp G., Obenauer-Kutner L. J., Duan J., Han M. A., Witchev-Lakshmanan L. C., and Grace M. J. (2005) Comparative thermal stabilities of recombinant adenoviruses and hexon protein. *Biochim Biophys Acta* **1726**, 138-151.

Abstract: Differential scanning calorimetry was used to identify the thermal stability profile of the replication deficient and protein IX deleted recombinant adenovirus type 5 that contains the p53 transgene (rAd/p53) in phosphate buffered saline (vPBS) or 10% glycerol (TRIS/phosphate buffer). The wildtype adenovirus (Ad/WT) and purified hexon protein (major capsid protein) were also evaluated in 10% glycerol (TRIS/phosphate buffer) as controls. The thermal profile of rAd/p53 revealed three endothermic transitions (T_1 , T_2 and T_3) occurring between 25 degrees C and 90 degrees C. T_1 , which occurred at 46.7 degrees C in vPBS and 49.4 degrees C in TRIS/PO₄ 10% glycerol buffer, was irreversible following repeated scanning and attributed to the degradation of the intact vector. The latter two endothermic transitions, T_2 and T_3 , occurring at 69 degrees C and 78 degrees C, respectively, corresponded with the two transitions of purified hexon in temperature and amount of heat absorbed. The thermal profile of Ad/WT revealed four endothermic transitions at 51.5 degrees C (T_1), 70.5 degrees C (T_2A), 73.6 degrees C (T_2B), and 77.4

degrees C (T3). The higher temperature of degradation as well as additional transition was attributed to the presence of protein IX associated with the hexon. The positions and excess molar heat capacities of the intact rAds were found to be affected by pH, glycerol, vector concentration and the presence or absence of protein IX in the capsid. Irreversibility of T1 implied that the degradation of the intact virus may follow first-order kinetics. The thermal scan rate dependence of T1 further confirmed that degradation of the intact virus may be first-order. The apparent activation energies for the degradation of the intact vectors were determined from the scan rate dependence of T1 and shown to be affected by protein IX in the capsid and solution conditions. Analysis of rAd samples incubated at 45 degrees C by Field Emission Electron Microscopy (FESEM) confirmed that loss of single particles was first-order. Although aggregates were observed in the samples, degradation appeared to be the dominant reaction leading to disappearance of single virions from the aqueous matrix. Based on thermal and FESEM analysis, an empirical model was proposed that accounted for the disappearance of single rAd particles. At or near T1, degradation of rAd particles followed a unidirectional, pseudo-first order reaction. However, at lower temperatures, disappearance of single virions resulted from competing irreversible degradation and aggregation reactions.

Jankowska E., Wiczak W., and Grzonka Z. (2004) Thermal and guanidine hydrochloride-induced denaturation of human cystatin C. *Eur Biophys J* **33**, 454-461.

Abstract: Wild-type human cystatin C is directly involved in pathological fibrils formation, leading to hemorrhage, dementia and eventually death of people suffering from cerebral amyloid angiopathy. Some studies on cystatin C oligomerization have been already done but some points are still unclear. In order to learn more about this important process, we have investigated thermal and chemical (guanidine hydrochloride-induced) denaturation of human cystatin C. Studies performed using tryptophan fluorescence, calorimetry, circular dichroism and Fourier transform infrared spectroscopy demonstrate that neither chemical nor thermal denaturation of hCC are simple two-state events. One recognized intermediate form was dimeric cystatin C, whose appearance was preceded mainly by changes in the L2 binding loop. The other form occurred only in the chemical denaturation process and was characterized by partially recovered interactions maintaining the protein tertiary structure. Our studies also strongly indicate that the beta-structural motif of cystatin C is directly implicated in formation of temperature-induced aggregates.

Jiang G., Woo B. H., Kang F., Singh J., and DeLuca P. P. (2002) Assessment of protein release kinetics, stability and protein polymer interaction of lysozyme encapsulated poly(D,L-lactide-co-glycolide) microspheres. *J Control Release* **79**, 137-145.

Abstract: Using lysozyme as a model protein, this study investigated protein stability, protein-polymer interaction in different release media and their influence on protein release profile and in vitro--in vivo correlation. Lysozyme was microencapsulated into PLGA 50:50 by a double emulsion--solvent extraction/evaporation method. Protein stability, protein-PLGA adsorption and protein in vitro release were studied in various test media. Differential scanning calorimetry analysis showed lysozyme to be most conformationally stable in pH 4.0 acetate buffer with highest Tm at 77.2 degree C and $\Delta H(\text{cal})$ 83.1 kcal/mol. Lysozyme exhibited good stability in pH 2.5 glycine buffer with Tm at 63.8 degree C and $\Delta H(\text{cal})$ 69.9 kcal/mol. In pH 7.4 phosphate-buffered saline (PBS), lysozyme showed a trend toward aggregation when the temperature was elevated. When PLGA polymer was incubated with lysozyme in the various buffers, adsorption was found to occur in PBS only. The adsorption severely limited the amount of lysozyme available for release from microspheres, resulting in slow and incomplete release in PBS. In contrast, the release of the microspheres in acetate and glycine buffers was complete within 40 and 70 days, respectively. Radiolabeled lysozyme blood levels in rats from the microspheres correlated qualitatively well with in vitro release in glycine buffer as a release medium. This study suggests that protein stability and adsorption are critical factors controlling protein release kinetics and in vitro--in vivo correlation of PLGA microspheres.

Jiao W., Qian M., Li P., Zhao L., Chang Z. (2005) The essential role of the flexible termini in the temperature-responsiveness of the oligomeric state and chaperone-like activity for the polydisperse small heat shock protein IbpB from *Escherichia coli*. *J Mol Biol.* **347**,871-84.

Abstract: Small heat shock proteins (sHSPs) represent an abundant and ubiquitous family of molecular chaperones that are believed to prevent irreversible aggregation of other cellular proteins under stress conditions. One of the most prominent features of sHSPs is that they exist as homo-oligomers. Examples of both monodisperse and polydisperse oligomers are found within this family. The small heat shock

inclusion-body binding protein B (IbpB) of *Escherichia coli*, originally discovered as a component of inclusion bodies, exhibits a pronounced polydispersity in its oligomeric state. This research was performed to elucidate the temperature effect on the oligomeric state and chaperone-like activity of the polydisperse IbpB oligomers, as well as the structural basis for such a temperature effect. The data presented here demonstrate that the large oligomers of IbpB progressively dissociate into smaller ones at increasing heat-shock temperatures, accompanied by a notable enhancement of chaperone-like activities. The secondary structure, enriched mainly by beta-strands, is slightly changed with such temperature increases. The dimeric building blocks, which seem to be highly stable, act as the functional unit of IbpB. Limited proteolysis was used to identify the susceptible sites in IbpB that may compose the subunit interfaces, which indicated that the 11 residues at both the N and the C terminus are highly flexible and the removal of each will lead to the formation of dimers, as well as the disappearance of chaperone-like activities. Truncation of 11 residues from either end, using recombinant DNA technology, also led to the formation of dimeric mutant IbpB proteins lacking chaperone-like activities. Taken together, the flexible termini appear to be essential for small heat shock protein IbpB to generate various temperature-responsive oligomers, which exhibit various levels of chaperone-like activities, by interlinking or separating the dimer building blocks.

Kameoka D., Masuzaki E., Ueda T. and Imoto T. (2007) Effect of buffer species on the unfolding and the aggregation of humanized IgG. *J Biochem (Tokyo)* **142**, 383-391.

Abstract: The aggregation propensity of humanized antibody after heat treatment is evaluated in the presence of six buffer species. The comparison under equivalent pH showed high aggregation propensity on phosphate and citrate buffer. In contrast, 2-(N-Morpholino) ethane sulfonate (MES), 3-(N-Morpholino) propane sulfonate (MOPS), acetate and imidazole buffer showed lower aggregation propensity than the above two buffers. Meanwhile, unfolding temperature evaluated by differential scanning calorimetry measurement was not altered among these buffer species. The light scattering analysis suggested that heat-denatured intermediate was aggregated slightly on MES and acetate buffer. Therefore, it was found that the different aggregation propensity among buffer species was caused from the aggregation propensity of heat-denatured intermediate rather than the unfolding temperature. Furthermore, it was revealed that the aggregation dependency on buffer species is accounted for by the specific molecular interaction between buffer and IgG, rather than the ionic strength. On the contrary, on the analyses of unfolding and aggregation propensity by molecular dissection of IgG into Fab and Fc fragments, aggregation propensity of Fc fragment on MES, acetate and phosphate buffer was almost the same as whole IgG. From the above results, it was suggested that the specific interaction between buffer molecule and Fc domain of IgG was involved in the aggregation propensity of heat-denatured IgG.

Kar K., Amin P., Bryan M. A., Persikov A. V., Mohs A., Wang Y. H., and Brodsky B. (2006) Self-association of collagen triple helix peptides into higher order structures. *J Biol Chem* **281**, 33283-33290.

Abstract: Interest in self-association of peptides and proteins is motivated by an interest in the mechanism of physiologically higher order assembly of proteins such as collagen as well as the mechanism of pathological aggregation such as beta-amyloid formation. The triple helical form of (Pro-Hyp-Gly)(10), a peptide that has proved a useful model for molecular features of collagen, was found to self-associate, and its association properties are reported here. Turbidity experiments indicate that the triple helical peptide self-assembles at neutral pH via a nucleation-growth mechanism, with a critical concentration near 1 mM. The associated form is more stable than individual molecules by about 25 degrees C, and the association is reversible. The rate of self-association increases with temperature, supporting an entropically favored process. After self-association, (Pro-Hyp-Gly)(10) forms branched filamentous structures, in contrast with the highly ordered axially periodic structure of collagen fibrils. Yet a number of characteristics of triple helix assembly for the peptide resemble those of collagen fibril formation. These include promotion of fibril formation by neutral pH and increasing temperature; inhibition by sugars; and a requirement for hydroxyproline. It is suggested that these similar features for peptide and collagen self-association are based on common lateral underlying interactions between triple helical molecules mediated by hydrogen-bonded hydration networks involving hydroxyproline.

Kar K. and Kishore N. (2007) Enhancement of thermal stability and inhibition of protein aggregation by osmolytic effect of hydroxyproline. *Biopolymers* **87**, 339-351.

Abstract: A combination of spectroscopic, calorimetric, and microscopic studies to understand the effect of hydroxyproline on the thermal stability, conformation, biological activity, and aggregation of proteins has been investigated. Significantly increased protein stability and suppression of aggregation is achieved in the presence of hydroxyproline. For example, exceptional increase in the thermal stability of lysozyme up to 26.4 degrees C and myoglobin up to 31.8 degrees C is obtained in the presence of hydroxyproline. The increased thermal stability of the proteins is observed to be accompanied with significant rise of the catalytic activity. Hydroxyproline is observed to prevent lysozyme fibril formation in vitro. Fluorescence and circular dichroism studies indicate induction of tertiary structures of the studied proteins in the presence of hydroxyproline. Preferential hydration of the native state is found to be crucial for the mechanism of protein stabilization by hydroxyproline. We compared the effect of hydroxyproline to that of proline and observed similar increase in the activity and suppression of protein aggregation. The results demonstrate the use of hydroxyproline as a protein stabilizer and in the prevention of protein aggregation and fibril formation.

Katayama D. S., Nayar R., Chou D. K., Valente J. J., Cooper J., Henry C. S., Vander Velde D. G., Villarete L., Liu C. P., and Manning M. C. (2006) Effect of buffer species on the thermally induced aggregation of interferon-tau. *J Pharm Sci* **95**, 1212-1226.

Abstract: It is now becoming apparent that a common pathway of protein aggregation involves the unimolecular structural rearrangement from the native state to a slightly expanded aggregation-competent species. It is the goal of this study to understand the aggregation and the effects of buffer on the stability of IFN-tau. In this study, the thermally-induced aggregation of interferon-tau (IFN-tau) is described. By monitoring the aggregation rate in the presence of increasing amounts of sucrose, the relative change in surface area (ΔA) for conversion to the aggregation-competent state can be determined. Under conditions of pH 7 and in 20 mM buffer, the protein displays different aggregation rates depending on the nature of the buffer species. The protein aggregates mostly quickly in phosphate buffer, slower in the presence of Tris and slowest in the presence of histidine. The largest value for ΔA occurs for the histidine-containing samples, where aggregation proceeds via a slightly expanded aggregation competent state with a surface area increase of 7.6%. Furthermore, it appears that histidine binds to the native state of IFN-tau, thereby stabilizing the native state and retarding aggregation. Measurement of the second virial coefficient, $B(22)$, for different formulations indicates that inclusion of histidine has only a small effect on repulsion between protein molecules, suggesting that colloidal stabilization is not the dominant mechanism for stabilization of IFN-tau. This study represents the first detailed biophysical study of specific buffer-induced stabilization, resulting in shifting the equilibrium towards the native state and away from the expanded aggregation-competent species.

Kato A., Nakamura S., Ban M., Azakami H., and Yutani K. (2000) Enthalpic destabilization of glycosylated lysozymes constructed by genetic modification. *Biochim Biophys Acta* **1481**, 88-96.

Abstract: To understand the role of polyglycosylation in protein stability, the thermodynamic changes in the denaturation of various polymannosyl lysozyme mutants (R21T, G49N, R21T/G49N) constructed by genetic modification were analyzed using differential scanning calorimetry (DSC). The denaturation temperature and the enthalpy change for unfolding of the lysozymes were reduced with an increase in the length of the polymannose chain and the number of binding sites to a protein, although the polymannosyl lysozymes revealed apparent heat stability in that no aggregation was observed and the enzymatic activity was conserved under conditions in which the wild-type lysozyme coagulated [S. Nakamura et al., *J. Biol. Chem.* 268 (1993) 12706-12712]. The reversibility of the denaturation of polymannosyl lysozymes was observed in the DSC curves obtained by reheating after heat denaturation, while it was not observed for the wild-type lysozyme. Based on these results, the polymannosyl lysozyme seems to easily refold due to the excellent reversibility of denaturation, despite the decreases in the enthalpic stabilization due to the strain in the protein molecule by the introduction of a polysaccharide chain.

Kegel W. K. and van der S. P. (2006) Physical regulation of the self-assembly of tobacco mosaic virus coat protein. *Biophys J* **91**, 1501-1512.

Abstract: We present a statistical mechanical model based on the principle of mass action that explains the main features of the in vitro aggregation behavior of the coat protein of tobacco mosaic virus (TMV). By comparing our model to experimentally obtained stability diagrams, titration experiments, and calorimetric data, we pin down three competing factors that regulate the transitions between the different kinds of

aggregated state of the coat protein. These are hydrophobic interactions, electrostatic interactions, and the formation of so-called "Caspar" carboxylate pairs. We suggest that these factors could be universal and relevant to a large class of virus coat proteins.

Khanova H. A., Markossian K. A., Kurganov B. I., Samoilov A. M., Kleimenov S. Y., Levitsky D. I., Yudin I. K., Timofeeva A. C., Muranov K. O., and Ostrovsky M. A. (2005) Mechanism of Chaperone-like Activity. Suppression of Thermal Aggregation of beta(L)-Crystallin by alpha-Crystallin. *Biochemistry* **44**, 15480-15487.

Abstract: Thermal denaturation and aggregation of beta(L)-crystallin from bovine lens have been studied using differential scanning calorimetry (DSC) and dynamic light scattering (DLS). According to the DLS data, the distribution of the beta(L)-crystallin aggregates by their hydrodynamic radius ($R(h)$) remains monomodal to the point of precipitating aggregates (sodium phosphate, pH 6.8; 100 mM NaCl; 60 degrees C). The size of the start aggregates ($R(h,0)$) and duration of the latent stage ($t(0)$) leading to the formation of the start aggregates have been determined from the light scattering intensity versus the hydrodynamic radius plots and the dependences of $R(h)$ on time. The $R(h,0)$ value remains constant at variation of the beta(L)-crystallin concentration, whereas the $t(0)$ value increases with diminishing beta(L)-crystallin concentration. The suppression of beta(L)-crystallin aggregation by alpha-crystallin is connected with the decrease in the $R(h,0)$ value and increase in the $t(0)$ value. In the presence of alpha-crystallin the aggregate population is split into two components. The first component is represented by stable aggregates whose size remains constant in time. The aggregates of the other kind grow until they reach the size characteristic of aggregates prone to precipitation. The DSC data show that alpha-crystallin has no appreciable influence on thermal denaturation of beta(L)-crystallin.

Khanova H. A., Markossian K. A., Kleimenov S. Y., Levitsky D. I., Chebotareva N. A., Golub N. V., Asryants R. A., Muronetz V. I., Saso L., Yudin I. K., Muranov K. O., Ostrovsky M. A. and Kurganov B. I. (2007) Effect of alpha-crystallin on thermal denaturation and aggregation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. *Biophys Chem* **125**, 521-531.

Abstract: The study of thermal denaturation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in the presence of alpha-crystallin by differential scanning calorimetry (DSC) showed that the position of the maximum on the DSC profile ($T(max)$) was shifted toward lower temperatures with increasing alpha-crystallin concentration. The diminishing GAPDH stability in the presence of alpha-crystallin has been explained assuming that heating of GAPDH induces dissociation of the tetrameric form of the enzyme into dimers interacting with alpha-crystallin. The dissociation of the enzyme tetramer was shown by sedimentation velocity at 45 degrees C. Suppression of thermal aggregation of GAPDH by alpha-crystallin was studied by dynamic light scattering under the conditions wherein temperature was elevated at a constant rate. The construction of the light scattering intensity versus the hydrodynamic radius ($R(h)$) plots enabled estimating the hydrodynamic radius of the start aggregates ($R(h,0)$). When aggregation of GAPDH was studied in the presence of alpha-crystallin, the start aggregates of lesser size were observed.

Kim Y. S., Randolph T. W., Manning M. C., Stevens F. J., and Carpenter J. F. (2003) Congo red populates partially unfolded states of an amyloidogenic protein to enhance aggregation and amyloid fibril formation. *J Biol Chem* **278**, 10842-10850.

Abstract: Congo red (CR) has been reported to inhibit or enhance amyloid fibril formation by several proteins. To gain insight into the mechanism(s) for these apparently paradoxical effects, we studied as a model amyloidogenic protein, a dimeric immunoglobulin light chain variable domain. With a range of molar ratios of CR, i.e. $r = [CR]/[protein\ dimer]$, we investigated the aggregation kinetics, conformation, hydrogen-deuterium exchange, and thermal stability of the protein. In addition, we used isothermal titration calorimetry to characterize the thermodynamics of CR binding to the protein. During incubation at 37 degrees C or during thermal scanning, with CR at $r = 0.3, 1.3,$ and 4.8 , protein aggregation was greatly accelerated compared with that measured in the absence of the dye. In contrast, with CR at $r = 8.8$, protein unfolding was favored over aggregation. The aggregates formed with CR at $r = 0$ or 0.3 were typical amyloid fibrils, but mixtures of amyloid fibrils and amorphous aggregates were formed at $r = 1.3$ and 4.8 . CR decreased the apparent thermal unfolding temperature of the protein. Furthermore, CR perturbed the tertiary structure of the protein without significantly altering its secondary structure. Consistent with this result, CR also increased the rate of hydrogen-deuterium exchange by the protein. Isothermal titration calorimetry showed that CR binding to the protein was enthalpically driven, indicating that binding was

mainly the result of electrostatic interactions. Overall, these results demonstrate that at low concentrations, CR binding to the protein favors a structurally perturbed, aggregation-competent species, resulting in acceleration of fibril formation. At high CR concentration, protein unfolding is favored over aggregation, and fibril formation is inhibited. Because low concentrations of CR can promote amyloid fibril formation, the therapeutic utility of this compound or its analogs to inhibit amyloidoses is questionable.

Koppelman S. J., van Koningsveld G. A., Knulst A. C., Gruppen H., Pigmans I. G., and de Jongh H. H. (2002) Effect of heat-induced aggregation on the IgE binding of patatin (Sol t 1) is dominated by other potato proteins. *J Agric Food Chem* **50**, 1562-1568.

Abstract: The interaction of the major potato allergen patatin, Sol t 1, with IgE was investigated on a quantitative level as a function of heat treatment at different temperatures. On the basis of a number of publications, potato is considered to be a heat-labile allergen, but the molecular explanation for this behavior was not given. In this work, heat treatment of patatin in the absence and presence of other potato proteins mimicking the proteinaceous environment of the potato was studied. Using far-UV circular dichroism spectroscopy, tryptophan fluorescence spectroscopy, and differential scanning calorimetry, the molecular transitions during heating of patatin were investigated. It was found that as long as patatin is not aggregated, denaturation of patatin on a secondary or tertiary folding level is reversible with only a minor effect on the IgE affinity. Aggregation of patatin results in a nonreversible unfolding and a concomitant important decrease in affinity for IgE (25-fold). Aggregation of patatin in the presence of other potato proteins results in a less condensed aggregate compared to the situation of isolated patatin, resulting in a more pronounced decrease of affinity for IgE (110-fold). It is concluded that the heat lability of patatin-IgE interaction is explained by aggregation of patatin with other potato proteins rather than by denaturation of patatin itself.

Krumova S. B., Todinova S. J., Busheva M. C., and Taneva S. G. (2005) Kinetic nature of the thermal destabilization of LHCII macroaggregates. *J Photochem Photobiol B* **78**, 165-170.

Abstract: The main light-harvesting chl a/b pigment-protein complex of photosystem II (LHCII) in isolated state forms macroaggregates with different ultrastructure and lipid content [I. Simidjiev, V. Barzda, L. Mustardy, G. Garab, *Anal. Biochem.* 250 (1997) 169-175]. The thermodynamic stability of highly delipidated tightly bound LHCII macroaggregates is studied by differential scanning calorimetry and fluorescence spectroscopy. The calorimetric profile of LHCII is asymmetric, the denaturation transition is taking place at around 72 degrees C. A shoulder, which overlaps with the main denaturation transition, appears around 58 degrees C. The denaturation temperature strongly depends on the scanning rate indicating the kinetic nature of the thermal destabilization of LHCII macroaggregates. The fluorescence data prove that the thermal denaturation of LHCII is an irreversible and kinetically controlled process.

Kueltzo L. A. and Middaugh C. R. (2003) Structural characterization of bovine granulocyte colony stimulating factor: effect of temperature and pH. *J Pharm Sci* **92**, 1793-1804.

Abstract: The protein bovine granulocyte colony stimulating factor (bGCSF) was studied in solution as a function of pH (2-7) and temperature (10 degrees -90 degrees C) using fluorescence, circular dichroism, and Fourier transform infrared spectroscopies, as well as differential scanning calorimetry and optical density as a measurement of aggregation. bGCSF possesses significant conformational lability under the solution conditions examined. Under all pH conditions examined, a major conformational change is observed as a function of temperature at 50 degrees -60 degrees C, although the magnitude and precise temperature at which this occurs varies with pH. Three major conformations are adopted with changing pH. One is observed at pH 2 and 3, a second at pH 4, and a third at pH 5-7. At low pH (2-3), bGCSF adopts a molten globule-like conformation at moderate temperatures (25 degrees -45 degrees C), whereas at pH 4 the protein appears to form a non-molten globule extended conformation. The use of this type of study as complementary data for protein phase diagram development as well as the relationship between the conformational lability demonstrated by bGCSF and that observed for recombinant human granulocyte colony stimulating factor and other similar cytokines is discussed.

Kurganov B. I., Rafikova E. R., and Dobrov E. N. (2002) Kinetics of thermal aggregation of tobacco mosaic virus coat protein. *Biochemistry (Mosc)* **67**, 525-533.

Abstract: The kinetics of thermal aggregation of coat protein (CP) of tobacco mosaic virus (TMV) have been studied at 42 and 52 degrees C in a wide range of protein concentrations, [P]₀. The kinetics of

aggregation were followed by monitoring the increase in the apparent absorbance (A) at 320 nm. At 52 degrees C the kinetic curves may be approximated by the exponential law in the range of TMV CP concentrations from 0.02 to 0.30 mg/ml, the first order rate constant being linearly proportional to [P]0 (50 mM phosphate buffer, pH 8.0). The analogous picture was observed at 42 degrees C in the range of TMV CP concentrations from 0.01 to 0.04 mg/ml (100 mM phosphate buffer, pH 8.0). At higher TMV CP concentrations the time of half-conversion approaches a limiting value with increasing [P]0 and at sufficiently high protein concentrations the kinetic curves fall on a common curve in the coordinates $[A/A(\text{lim}); t]$ (t is time and A(lim) is the limiting value of A at $t \rightarrow \infty$). According to a mechanism of aggregation of TMV CP proposed by the authors at rather low protein concentrations the rate of aggregation is limited by the stage of growth of aggregate, which proceeds as a reaction of the pseudo-first order, whereas at rather high protein concentrations the rate-limiting stage is the stage of protein molecule unfolding.

La Rosa C., Milardi D., Amato E., Pappalardo M., and Grasso D. (2005) Molecular mechanism of the inhibition of cytochrome c aggregation by Phe-Gly. *Arch Biochem Biophys* **435**, 182-189.

Abstract: Experimental and computational studies suggest that few general principles govern protein/protein interactions and aggregation. The knowledge of these rules may be exploited to design peptides that are able to interfere with the self-assembly and aggregation of proteins. This work is aimed to verify the validity of this hypothesis by investigating the interaction of cytochrome c with Phe and Gly amino acids, Ala-His (carnosine), and two water-soluble dipeptides Phe-Gly and Gly-Phe. The combined use of (1)H NMR, MD, and DSC has shown that: (i) at neutral pH, only Phe-Gly is able to prevent the thermally induced aggregation of cytochrome c; (ii) Phe-Gly interacts with Gly45 and Phe46 residues of the protein, either when the protein is in the folded or in the unfolded state; and (iii) the interaction of Phe-Gly with cytochrome c is sequence-dependent. These results support the hypothesis that the basic principles that describe protein aggregation can be used for the design of peptides with antiaggregating properties.

Lambeth R. H., Ramakrishnan S., Mueller R., Poziemski J. P., Miguel G. S., Markoski L. J., Zukoski C. F., and Moore J. S. (2006) Synthesis and aggregation behavior of thermally responsive star polymers. *Langmuir* **22**, 6352-6360.

Abstract: To mimic the three-dimensional (3-D) globular architecture resulting from the precise positioning of hydrophobic/hydrophilic domains (blocks) of naturally occurring proteins, water-soluble linear and star homopolymers of N,N'-dimethylacrylamide (DMA) were synthesized with prescribed molecular weights via reversible addition-fragmentation chain transfer (RAFT) polymerization and subsequently used as macro chain transfer agents for block copolymerization with N-isopropylacrylamide (NIPAM). For the star block copolymers, the interior block consisted of NIPAM while the exterior block was DMA. Since polyNIPAM thermally switches from hydrophilic to hydrophobic, the 3-D solution conformations of the polymers were studied as a function of temperature using differential scanning calorimetry (DSC), static light scattering (SLS), and dynamic light scattering (DLS). The polymers were observed to form monodisperse aggregates in an aqueous pH 4 buffer solution when heated above the lower critical solution temperature (LCST) of polyNIPAM. The temperature at which the polymers aggregated and the size of the aggregates were dependent on the NIPAM block length and the core architecture. A simple model based on an optimal area per headgroup was used to analyze our experimental findings and was useful for predicting the final size and molecular weight of the aggregates formed.

Lee R. C., Despa F., Guo L., Betala P., Kuo A., and Thiyagarajan P. (2006) Surfactant copolymers prevent aggregation of heat denatured lysozyme. *Ann Biomed Eng* **34**, 1190-1200.

Abstract: We investigated the ability of certain triblock copolymer surfactant poloxamers of the form polyethylene oxide-polypropylene oxide-polyethylene oxide (PEO-PPO-PEO), to prevent formation of stable aggregates of heat denatured hen egg lysozyme. Differential scanning calorimetry (DSC) and synchrotron small angle x-ray scattering (SAXS) experiments were performed to study the thermodynamics and solution structures of lysozyme at temperatures between 20 and 90 degrees C in the presence and absence of poloxamers with various molecular weights (8.4-14.3 kDa), but similar hydrophile/hydrophobe (PEO:PPO) ratio of 80%. Poloxmer 188 was found to be very effective in preventing aggregation of heat denatured lysozyme and those functioned as a synthetic surfactant, thus enabling them to refold when the conditions become optimal. For comparison, we measured the ability of 8 kDa polyethylene glycol (PEG) to prevent lysozyme aggregation under same conditions. The results of these studies suggest that

poloxamers are more efficient than PEG in preventing aggregation of heat denatured lysozyme. To achieve equivalence, more than an order of magnitude higher concentration of PEG concentration was needed. Apparently, the presence of a hydrophobic segment in the poloxamers increases their ability to target the hydrophobic region of the unfolded proteins and protect them from self association. Given their biocompatibility and the low concentrations at which they effectively facilitate refolding of denatured proteins, they may be useful in the treatment of burns and other conditions resulting in the denaturation of proteins.

Lendel C., Dincbas-Renqvist V., Flores A., Wahlberg E., Dogan J., Nygren P. A., and Hard T. (2004) Biophysical characterization of Z(SPA-1)--a phage-display selected binder to protein A. *Protein Sci* **13**, 2078-2088.

Abstract: Affibodies are a novel class of binding proteins selected from phagemid libraries of the Z domain from staphylococcal protein A. The Z(SPA-1) affibody was selected as a binder to protein A, and it binds the parental Z domain with micromolar affinity. In earlier work we determined the structure of the Z:Z(SPA-1) complex and noted that Z(SPA-1) in the free state exhibits several properties characteristic of a molten globule. Here we present a more detailed biophysical investigation of Z(SPA-1) and four Z(SPA-1) mutants with the objective to understand these properties. The characterization includes thermal and chemical denaturation profiles, ANS binding assays, size exclusion chromatography, isothermal titration calorimetry, and an investigation of structure and dynamics by NMR. The NMR characterization of Z(SPA-1) was facilitated by the finding that trimethylamine N-oxide (TMAO) stabilizes the molten globule conformation in favor of the fully unfolded state. All data taken together lead us to conclude the following: (1) The topology of the molten globule conformation of free Z(SPA-1) is similar to that of the fully folded structure in the Z-bound state; (2) the extensive mutations in helices 1 and 2 destabilize these without affecting the intrinsic stability of helix 3; (3) stabilization and reduced aggregation can be achieved by replacing mutated residues in Z(SPA-1) with the corresponding wild-type Z residues. This stabilization is better correlated to changes in helix propensity than to an expected increase in polar versus nonpolar surface area of the fully folded state.

Lepock J. R., Frey H. E., Heynen M. L., Senisterra G. A., and Warters R. L. (2001) The nuclear matrix is a thermolabile cellular structure. *Cell Stress Chaperones* **6**, 136-147.

Abstract: Heat shock sensitizes cells to ionizing radiation, cells heated in S phase have increased chromosomal aberrations, and both Hsp27 and Hsp70 translocate to the nucleus following heat shock, suggesting that the nucleus is a site of thermal damage. We show that the nuclear matrix is the most thermolabile nuclear component. The thermal denaturation profile of the nuclear matrix of Chinese hamster lung V79 cells, determined by differential scanning calorimetry (DSC), has at least 2 transitions at $T_m = 48$ degrees C and 55 degrees C with an onset temperature of approximately 40 degrees C. The heat absorbed during these transitions is 1.5 cal/g protein, which is in the range of enthalpies for protein denaturation. There is a sharp increase in 1-anilinonaphthalene-8-sulfonic acid (ANS) fluorescence with $T_m = 48$ degrees C, indicating increased exposure of hydrophobic residues at this transition. The $T_m = 48$ degrees C transition has a similar T_m to those predicted for the critical targets for heat-induced clonogenic killing ($T_m = 46$ degrees C) and thermal radiosensitization ($T_m = 47$ degrees C), suggesting that denaturation of nuclear matrix proteins with $T_m = 48$ degrees C contribute to these forms of nuclear damage. Following heating at 43 degrees C for 2 hours, Hsc70 binds to isolated nuclear matrices and isolated nuclei, probably because of the increased exposure of hydrophobic domains. In addition, approximately 25% of exogenous citrate synthase also binds, indicating a general increase in aggregation of proteins onto the nuclear matrix. We propose that this is the mechanism for increased association of nuclear proteins with the nuclear matrix observed in nuclei isolated from heat-shocked cells and is a form of indirect thermal damage.

Liu Y., Breslauer K., and Anderson S. (1997) "Designing out" disulfide bonds: thermodynamic properties of 30-51 cystine substitution mutants of bovine pancreatic trypsin inhibitor. *Biochemistry* **36**, 5323-5335.

Abstract: We have used a combination of spectroscopic and calorimetric techniques to assess the thermodynamic and extrathermodynamic consequences of paired amino acid substitutions at positions 30 and 51 in bovine pancreatic trypsin inhibitor (BPTI). Correctly folded, wild type BPTI contains a disulfide at the 30-51 positions, with the nonbackbone atoms of this cystine being relatively solvent inaccessible. Mutants missing this buried 30-51 disulfide adopt a conformation very similar to that of the native state of wild type BPTI (Eigenbrot et al., 1990, 1992), although they are severely destabilized relative to the wild

type molecule (Hurle et al., 1990). We have conducted a systematic effort to find the energetically most favorable substitution for this buried 30-51 disulfide in BPTI. To this end, we have studied and characterized the thermally induced and guanidine hydrochloride-induced denaturation transitions for a family of mutants in which the amino acid residue(s) at positions 30 and/or 51 have been systematically altered. Specifically, we studied the unfolding transitions of the following series of residue 30/residue 51 paired substitution mutants: C30A/C51A, C30V/C51A, C30G/C51A, C30S/C51A, C30T/C51A, C30A/C51S, C30S/C51S, and C30G/C51M. For this series of mutants, comparisons between the relative stabilization free energies, derived from analysis of the denaturation profiles, allow us to reach the following conclusions: (a) side chains containing polar moieties (Ser and Thr) are destabilizing, with this effect being position dependent (i.e., a serine substitution is more destabilizing at position 51 than at position 30); (b) the destabilizing effects of two serine substitutions are approximately additive, suggesting that side chain-side chain hydrogen bonds between the two serine hydroxyl groups probably are weak or nonexistent; (c) the thermodynamic impact of a Gly30 substitution is consistent with a glycine-induced increase in the configurational entropy of the unfolded state; (d) the C30G/C51M mutant is highly destabilized relative to the C30A/C51A mutant despite the fact that, based on considerations of hydrophobicity and steric fit, substitution of a buried disulfide by Gly30 and Met51 would be expected to be optimal. Methionine may be particularly ill-suited as a buried disulfide substitute due to the large loss of side chain conformational entropy it undergoes during the transition from the unfolded to the native state. In the aggregate, our data provide insight into the residue-, position-, and context-dependent consequences on protein stability of "designing out" the buried 30-51 disulfide bond in the BPTI molecule. These data also suggest that a previously unrecognized component of disulfide bridge stabilization of proteins is the relatively minor penalty in side chain conformational entropy incurred by cystine residues during folding due to their severely restricted rotation even in the unfolded state.

Levitsky D. I., Pivovarova A. V., Mikhailova V. V. and Nikolaeva O. P. (2008) Thermal unfolding and aggregation of actin. *FEBS J* **275**, 4280-4295.

Abstract: Actin is one of the most abundant proteins in nature. It is found in all eukaryotes and plays a fundamental role in many diverse and dynamic cellular processes. Also, actin is one of the most ubiquitous proteins because actin-like proteins have recently been identified in bacteria. Actin filament (F-actin) is a highly dynamic structure that can exist in different conformational states, and transitions between these states may be important in cytoskeletal dynamics and cell motility. These transitions can be modulated by various factors causing the stabilization or destabilization of actin filaments. In this review, we look at actin stabilization and destabilization as expressed by changes in the thermal stability of actin; specifically, we summarize and analyze the existing data on the thermal unfolding of actin as measured by differential scanning calorimetry. We also analyze in vitro data on the heat-induced aggregation of actin, the process that normally accompanies actin thermal denaturation. In this respect, we focus on the effects of small heat shock proteins, which can prevent the aggregation of thermally denatured actin with no effect on actin thermal unfolding. As a result, we have proposed a mechanism describing the thermal denaturation and aggregation of F-actin. This mechanism explains some of the special features of the thermal unfolding of actin filaments, including the effects of their stabilization and destabilization; it can also explain how small heat shock proteins protect the actin cytoskeleton from damage caused by the accumulation of large insoluble aggregates under heat shock conditions

Lommer B. S. and Luo M. (2002) Structural plasticity in influenza virus protein NS2 (NEP). *J Biol Chem* **277**, 7108-7117.

Abstract: The cellular nuclear transport machinery relies on the assembly of specialized transport complexes between soluble transport receptors, transport substrates, and additional accessory proteins. This study focuses on the structural characteristics of influenza virus protein NS2 (NEP), which interacts with the nuclear export machinery during viral replication, and has been proposed to act as an adapter molecule between the nuclear export machinery and the viral ribonucleoprotein complex. For this purpose, we have purified recombinant NS2 under nondenaturing conditions, and have investigated its structure and aggregation state using optical spectroscopy, differential scanning calorimetry, as well as hydrodynamic techniques. Our results indicate that isolated NS2 exists as a monomer in solution, and adopts a compact, but very flexible conformation, which shows characteristics of the molten globule state under near physiological conditions. Proteolytic sensitivity suggests that, despite its overall plasticity, the structure of NS2 is heterogeneous. While the C terminus of the protein adopts a relatively rigid conformation, its N

terminus, which is recognized by the nuclear export machinery, exists in a highly mobile and exposed state. It is proposed that the flexibility observed in the nuclear export domain of NS2 is an important element in the recognition of substrate proteins by the nuclear export machinery.

Magdaleno L., Gasset M., Varea J., Schambony A. M., Urbanke C., Raida M., Topfer-Petersen E., and Calvete J. J. (1997) Biochemical and conformational characterisation of HSP-3, a stallion seminal plasma protein of the cysteine-rich secretory protein (CRISP) family. *FEBS Lett* **420**, 179-185.

Abstract: HSP-3 is a member of the cysteine-rich secretory protein (CRISP) family from stallion seminal plasma. We report a large-scale purification protocol for native HSP-3. This protein is a non-glycosylated polypeptide chain with a pI of 8-9 and an isotope-averaged molecular mass of 24987 +/- 3 Da. The molecular mass of HSP-3, determined by equilibrium sedimentation, is 26 kDa, showing that the protein exists in solution as a monomer. The concentration of HSP-3 in the seminal plasma of different stallions ranged from 0.3 to 1.3 mg/ml. On average, 0.9-9 million HSP-3 molecules/cell coat the postacrosomal and mid-piece regions of an ejaculated, washed stallion spermatozoon, suggesting a role in sperm physiology. Conformational characterisation of purified HSP-3 was assessed by combination of circular dichroism and Fourier-transform infrared spectroscopies and differential scanning microcalorimetry. Based on secondary structure assignment, HSP-3 may belong to the alpha+beta class of proteins. Thermal denaturation of HSP-3 is irreversible and follows a non-two state transition characterised by a T_m of 64 degrees C, an enthalpy change of 75 kcal/mol, and a van 't Hoff enthalpy of 184 kcal/mol. Analysis of the spectroscopic and calorimetric data indicates the occurrence of aggregation of denatured HSP-3 molecules and suggests the monomer as the cooperative unfolding unit.

Markossian K. A., Khanova H. A., Kleimenov S. Y., Levitsky D. I., Chebotareva N. A., Asryants R. A., Muronetz V. I., Saso L., Yudin I. K., and Kurganov B. I. (2006) Mechanism of thermal aggregation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry* **45**, 13375-13384.

Abstract: Thermal denaturation and aggregation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been studied using differential scanning calorimetry (DSC), dynamic light scattering (DLS), and analytical ultracentrifugation. The maximum of the protein thermal transition (T_m) increased with increasing the protein concentration, suggesting that the denaturation process involves the stage of reversible dissociation of the enzyme tetramer into the oligomeric forms of lesser size. The dissociation of the enzyme tetramer was shown by sedimentation velocity at 45 degrees C. The DLS data support the mechanism of protein aggregation that involves a stage of the formation of the start aggregates followed by their sticking together. The hydrodynamic radius of the start aggregates remained constant in the temperature interval from 37 to 55 degrees C and was independent of the protein concentration (R(h,0) approximately 21 nm; 10 mM sodium phosphate, pH 7.5). A strict correlation between thermal aggregation of GAPDH registered by the increase in the light scattering intensity and protein denaturation characterized by DSC has been proved.

Markossian K. A., Golub N. V., Khanova H. A., Levitsky D. I., Poliansky N. B., Muranov K. O. and Kurganov B. I. (2008) Mechanism of thermal aggregation of yeast alcohol dehydrogenase I: role of intramolecular chaperone. *Biochim Biophys Acta* **1784**, 1286-1293.

Abstract: Kinetics of thermal aggregation of yeast alcohol dehydrogenase I (yADH) have been studied using dynamic light scattering at a fixed temperature (56 degrees C) and under the conditions where the temperature was elevated at a constant rate (1 K/min). The initial parts of the dependences of the hydrodynamic radius on time (or temperature) follow the exponential law. At rather high values of time splitting of the population of aggregates into two components occurs. It is assumed that such peculiarities of the kinetics of thermal aggregation of yADH are due to the presence of a sequence - YSGVCHTDLHAWHGDWP

Markov D. I., Pivovarova A. V., Chernik I. S., Gusev N. B. and Levitsky D. I. (2008) Small heat shock protein Hsp27 protects myosin S1 from heat-induced aggregation, but not from thermal denaturation and ATPase inactivation. *FEBS Lett* **582**, 1407-1412.

Abstract: We applied different methods, such as turbidity measurements, dynamic light scattering, differential scanning calorimetry and co-sedimentation assay, to analyze the interaction of small heat shock protein Hsp27 with isolated myosin head (myosin subfragment 1, S1) under heat-stress conditions. Upon heating at 43 degrees C, Hsp27 effectively suppresses S1 aggregation, and this effect is enhanced by

mutations mimicking Hsp27 phosphorylation. However, Hsp27 was unable to prevent thermal unfolding of myosin heads and to maintain their ATPase activity under heat-shock conditions

McGuffey M. K., Epting K. L., Kelly R. M., and Foegeding E. A. (2005) Denaturation and aggregation of three alpha-lactalbumin preparations at neutral pH. *J Agric Food Chem* **53**, 3182-3190.

Abstract: The denaturation and aggregation of reagent-grade (Sigmaalpha-La), ion-exchange chromatography purified (IEXalpha-La), and a commercial-grade (Calpha-La) alpha-lactalbumin were studied with differential scanning calorimetry (DSC), polyacrylamide gel electrophoresis, and turbidity measurement. All three preparations had similar thermal denaturation temperatures with an average of 63.7 degrees C. Heating pure preparations of alpha-lactalbumin produced three non-native monomer species and three distinct dimer species. This phenomenon was not observed in Calpha-La. Turbidity development at 95 degrees C (tau95 degrees C) indicated that pure preparations rapidly aggregate at pH 7.0, and evidence suggests that hydrophobic interactions drove this phenomenon. The Calpha-La required 4 times the phosphate or excess Ca²⁺ concentrations to develop a similar tau95 degrees C to the pure preparations and displayed a complex pH-dependent tau95 degrees C behavior. Turbidity development dramatically decreased when the heating temperature was below 95 degrees C. A mechanism is provided, and the interrelationship between specific electrostatic interactions and hydrophobic attraction, in relation to the formation of disulfide-bonded products, is discussed.

Meng G. T. and Ma C. Y. (2001) Fourier-transform infrared spectroscopic study of globulin from *Phaseolus angularis* (red bean). *Int J Biol Macromol* **29**, 287-294.

Abstract: The conformation of red bean globulin dispersions (approximately 10% in D₂O or deuterated phosphate buffer pD 7.4) under the influence of pH, chaotropic salts, protein structure perturbants, and heating conditions was studied by Fourier-transform infrared (FTIR) spectroscopy. The FTIR spectrum of red bean globulin showed major bands from 1682 to 1637 cm⁻¹ in the amide I' region, corresponding to the four types of secondary structures, i.e. beta-turns, beta-sheets, alpha-helix and random coils. At extreme pH conditions, there were changes in intensity in bands attributed to beta-sheet (1637 and 1618 cm⁻¹) and random coil (1644 cm⁻¹) structures, and shifts of these bands to lower or higher wave numbers, indicating changes in protein conformation. Chaotropic salts caused progressive increases in random coil structures and concomitant decreases in beta-sheet bands, following the lyotropic series of anions. In the presence of sodium dodecyl sulfate and ethylene glycol, pronounced increases in the random coil band were observed, accompanied by slight shifts of the beta-sheet band. Addition of dithiothreitol and N-ethylmaleimide did not cause marked changes in the FTIR spectra. Heating at increasing temperature led to progressive decreases in the intensity of the alpha-helix and beta-sheet bands and increases in random coil band intensity, leveling off at around 60 degrees C. The data suggest that re-organization of protein structure occurred at temperatures well below the denaturation temperature of red bean globulin (86 degrees C) as determined by differential scanning calorimetry. This was accompanied by pronounced increases in the intensity of the two intermolecular beta-sheet bands (1682 and 1619-1620 cm⁻¹) associated with the formation of aggregated strands at higher temperatures (80-90 degrees C). Increases in intensity of the aggregation bands were also observed in the heat-induced buffer-soluble and insoluble aggregates.

Mills E. N., Huang L., Noel T. R., Gunning A. P., and Morris V. J. (2001) Formation of thermally induced aggregates of the soya globulin beta-conglycinin. *Biochim Biophys Acta* **1547**, 339-350.

Abstract: The effect of ionic strength (I) on the formation of thermally induced aggregates by the 7S globular storage protein of soya, beta-conglycinin, has been studied using atomic force microscopy. Aggregates were only apparent when I > or = 0.1, and had a fibrous appearance, with a height (diameter) of 8-11 nm. At high ionic strength (I=1.0) the aggregates appeared to associate into clumps. When aggregate formation was studied at I=0.2, it was clear that aggregation only began at temperatures above the main thermal transition for the protein at 75 degrees C, as determined by differential scanning calorimetry. This coincided with a small change in secondary structure, as indicated by circular dichroism spectroscopy, suggesting that a degree of unfolding was necessary for aggregation to proceed. Despite prolonged heating the size of the aggregates did not increase indefinitely, suggesting that certain beta-conglycinin isoforms were able to act as chain terminators. At higher protein concentrations (1% w/v) the linear aggregates appeared to form large macroaggregates, which may be the precursors of protein gel formation. The ability of beta-conglycinin to form such distinctive aggregates is discussed in relation to the presence of acidic

inserts in certain of the beta-conglycinin subunits, which may play an important role in limiting aggregate length.

Misselwitz R., Hausdorf G., Welfle K., Hohne W. E., and Welfle H. (1995) Conformation and stability of recombinant HIV-1 capsid protein p24 (rp24). *Biochim Biophys Acta* **1250**, 9-18.

Abstract: Conformation and stability of the recombinant protein HIV-1 rp24 were analyzed by circular dichroism, fluorescence spectroscopy and differential scanning calorimetry under different solvent conditions. From circular dichroism measurements, HIV-1 rp24 at pH 5.8 can be classified as an all alpha-helical protein. A fluorescence maximum of about 330 nm indicates a predominantly hydrophobic environment of the five tryptophan residues. The GdnHCl-induced unfolding curves monitored by CD and fluorescence are sigmoidal and single phasic and the midpoints of transitions are independent on the protein concentration. For the calculation of free energy of unfolding ΔG_{H_2O} a 'two-state' model was applied. The calculated values are between 18 and 24 kJ/mol and thus on the lower limit of the conformational stability of globular proteins. Melting experiments at pH 5.8 are impaired by a strong irreversible aggregation at higher temperatures. However, at pH 3.0 and in the presence of 0.1% (w/v) octyl beta-glucopyranoside the melting curves show a large degree of reversibility with a T_m value of 38 degrees C and a molar enthalpy change ΔH_m of 218 kJ/mol. At pH < 2.5 HIV-1 rp24 can adopt a new conformation which is characterized by a high alpha-helical content, a strongly decreased CD in the aromatic region, a red-shift of the fluorescence spectrum and a strong binding of ANS. These spectral features of the acid-induced conformational state are similar to those obtained for molten globule-like folding states. HIV-1 rp24 unfolds cooperatively at pH 2.0 in the concentration range of about 1.5-3.0 M GdnHCl. The calculated values ΔG_{H_2O} at pH 2.0 of about 12 kJ/mol are significantly decreased in comparison to the ΔG_{H_2O} values of the protein at pH 5.8.

Mizutani K., Chen Y., Yamashita H., Hirose M., and Aibara S. (2006) Thermostabilization of ovotransferrin by anions for pasteurization of liquid egg white. *Biosci Biotechnol Biochem* **70**, 1839-1845.

Abstract: The effects of anions on the thermostability of ovotransferrin (oTf) were investigated. The temperature, $T(m)$, causing aggregation of oTf was measured in the presence or absence of anions, and the denaturation temperature, $T(m)$ (DSC), was also determined by differential scanning calorimetry (DSC) in the presence of the citrate anion. We found that some anions (phosphate, sulfate and citrate) raised temperature $T(m)$ of oTf by about 5-7 degrees C. However, neither sodium chloride nor sodium bicarbonate raised $T(m)$ by that much. Temperature $T(m)$ was increased by increasing the concentration of the citrate anion, and was in good agreement with denaturation temperature $T(m)$ (DSC), suggesting that denaturation of the oTf molecules resulted in aggregation of oTf. We also demonstrated that the anions, especially sulfate, repressed the heat-aggregation of liquid egg white. The Van't Hoff plot from the $T(m)$ and $\Delta H(d)$ values revealed that two anion-binding sites were concerned with heat stabilization. These binding sites may have been concerned with sulfate binding (not bicarbonate binding) that is found in the crystal structure of apo-form of oTf, since the bicarbonate anion did not raise $T(m)$.

Morel B., Casares S., and Conejero-Lara F. (2006) A single mutation induces amyloid aggregation in the alpha-spectrin SH3 domain: analysis of the early stages of fibril formation. *J Mol Biol* **356**, 453-468.

Abstract: The Src-homology region 3 domain of chicken alpha-spectrin (Spc-SH3) is a small two-state folding protein, which has never been described to form amyloid fibrils under any condition investigated so far. We show here that the mutation of asparagine 47 to alanine at the distal loop, which destabilises similarly the native and folding transition states of the domain, induces the formation of amyloid fibrils under mild acid conditions. Amyloid aggregation of the mutant is enhanced by the increase in temperature, protein concentration and NaCl concentration. The early stages of amyloid formation have been monitored as a function of time and temperature using a variety of biophysical methods. Differential scanning calorimetry experiments under conditions of amyloid formation have allowed the identification of different thermal transitions corresponding to conformational and aggregation processes as well as to the high-temperature disaggregation and unfolding of the amyloid fibrils. Aggregation is preceded by a rapid conformational change in the monomeric domain involving about 40% of the global unfolding enthalpy, considerable change in secondary structure, large loss of tertiary structure and exposure of hydrophobic patches to the solvent. The conformational change is followed by formation of a majority of oligomeric species with apparent hydrodynamic radius between 2.5 nm and 10nm, depending on temperature, together with the appearance and progressive growth of protofibrillar aggregates. After these early aggregation

stages, long and curved fibrils of up to several micrometers start to develop by elongation of the protofibrils. The calorimetric data indicate that the specific enthalpy of fibril disaggregation and unfolding is relatively low, suggesting a low density of interactions within the fibril structure as compared to the native protein and a main entropy contribution to the stability of the amyloid fibrils.

Naganathan A. N., Perez-Jimenez R., Sanchez-Ruiz J. M., and Munoz V. (2005) Robustness of downhill folding: guidelines for the analysis of equilibrium folding experiments on small proteins. *Biochemistry* **44**, 7435-7449.

Abstract: Previously, we identified the protein BBL as a downhill folder. This conclusion was based on the statistical mechanical analysis of equilibrium experiments performed in two variants of BBL, one with a fluorescent label at the N-terminus, and another one labeled at both ends. A recent report has claimed that our results are an artifact of label-induced aggregation and that BBL with no fluorescent labels and a longer N-terminal tail folds in a two-state fashion. Here, we show that singly and doubly labeled BBL do not aggregate, unfold reversibly, and have the same thermodynamic properties when studied under appropriate experimental conditions (e.g., our original conditions (1)). With an elementary analysis of the available data on the nonlabeled BBL (2), we also show that this slightly more stable BBL variant is not a two-state folder. We discuss the problems that led to its previous misclassification and how they can be avoided. Finally, we investigate the equilibrium unfolding of the singly labeled BBL with both ends protected by acetylation and amidation. This variant has the same thermodynamic stability of the nonlabeled BBL and displays all the equilibrium signatures of downhill folding. From all these observations, we conclude that fluorescent labels do not perturb the thermodynamic properties of BBL, which consistently folds downhill regardless of its stability and specific protein tails. The work on BBL illustrates the shortcomings of applying conventional procedures intended to distinguish between two-state and three-state folding models to small fast-folding proteins.

Nielsen A. D., Pusey M. L., Fuglsang C. C., and Westh P. (2003) A proposed mechanism for the thermal denaturation of a recombinant *Bacillus halmapalus* alpha-amylase--the effect of calcium ions. *Biochim Biophys Acta* **1652**, 52-63.

Abstract: The thermal stability of a recombinant alpha-amylase from *Bacillus halmapalus* alpha-amylase (BHA) has been investigated using circular dichroism spectroscopy (CD) and differential scanning calorimetry (DSC). This alpha-amylase is homologous to other *Bacillus* alpha-amylases where crystallographic studies have identified the existence of three calcium binding sites in the structure. Denaturation of BHA is irreversible with a T_m of approximately 89 degrees C and DSC thermograms can be described using a one-step irreversible model. A 5 degrees C increase in T_m in the presence of 10-fold excess CaCl₂ was observed. However, a concomitant increase in the tendency to aggregate was also observed. The presence of 30-40-fold excess calcium chelator (ethylenediaminetetraacetic acid (EDTA) or ethylene glycol-bis[beta-aminoethyl ether] N,N,N',N'-tetraacetic acid (EGTA)) results in a large destabilization of BHA, corresponding to about 40 degrees C lower T_m as determined by both CD and DSC. Ten-fold excess EGTA reveals complex DSC thermograms corresponding to both reversible and irreversible transitions, which probably originate from different populations of BHA/calcium complexes. Combined interpretation of these observations and structural information on homologous alpha-amylases forms the basis for a suggested mechanism underlying the inactivation mechanism of BHA. The mechanism includes irreversible thermal denaturation of different BHA/calcium complexes and the calcium binding equilibria. Furthermore, the model accounts for a temperature-induced reversible structural change associated with calcium binding.

Olah J., Orosz F., Keseru G. M., Kovari Z., Kovacs J., Hollan S., and Ovadi J. (2002) Triosephosphate isomerase deficiency: a neurodegenerative misfolding disease. *Biochem Soc Trans* **30**, 30-38.

Abstract: A number of neurodegenerative diseases are mediated by mutation-induced protein misfolding. The resulting genetic defects, however, are expressed in varying phenotypes. Of the several well-established glycolytic enzyme deficiencies, triosephosphate isomerase (TPI) deficiency is the only one in which haemolytic anaemia is coupled with progressive, severe neurological disorder. In a Hungarian family with severe decrease in TPI activity, two germ line-identical but phenotypically differing compound heterozygote brothers inherited two independent (Phe(240)-->Leu and Glu(145)-->stop codon) mutations. We have demonstrated recently [Orosz, Olah, Alvarez, Keseru, Szabo, Wagner, Kovari, Horanyi, Baroti, Martial, Hollan and Ovadi (2001) *Blood* **98**, 3106-3112] that the mutations of TPI explain in themselves

neither the severe decrease in the enzyme activity characteristic of TPI deficiency nor the enhanced ability of the mutant enzyme from haemolysate of the propositus to associate with subcellular particles. Here we present kinetic (flux analysis), thermodynamic (microcalorimetry and fluorescence spectroscopy), structural (in silico) and ultrastructural (immunoelectron microscopy) data for characterization of mutant isomerase structures and for the TPI-related metabolic processes in normal and deficient cells. The relationships between mutation-induced TPI misfolding and formation of aberrant protein aggregates are discussed.

Orlov V. N., Kust S. V., Kalmykov P. V., Krivosheev V. P., Dobrov E. N., and Drachev V. A. (1998) A comparative differential scanning calorimetric study of tobacco mosaic virus and of its coat protein ts mutant. *FEBS Lett* **433**, 307-311.

Abstract: The differential scanning calorimetry (DSC) 'melting curves' for virions and coat proteins (CP) of wild-type tobacco mosaic virus (strain U1) and for its CP ts mutant ts21-66 were measured. Strain U1 and ts21-66 mutant (two amino acid substitutions in CP: 121 --> T and D66 --> G) differ in the type of symptoms they induce on some host plants. It was observed that CP subunits of both U1 and ts21-66 at pH 8.0, in the form of small (3-4S) aggregates, possess much lower thermal stability than in the virions. Assembly into the virus particles resulted in a DSC melting temperature increase from 41 to 72 degrees C for U1 and from 38 to 72 degrees C for ts21-66 CP. In the RNA-free helical virus-like protein assemblies U1 and ts21-66 CP subunits had a thermal stability intermediate between those in 3-4S aggregates and in the virions. ts21-66 helical protein displayed a somewhat lower thermal stability than U1.

Orlov V. N., Arutyunyan A. M., Kust S. V., Litmanovich E. A., Drachev V. A., and Dobrov E. N. (2001) Macroscopic aggregation of tobacco mosaic virus coat protein. *Biochemistry (Mosc)* **66**, 154-162.

Abstract: The relationship between processes of thermal denaturation and heat-induced aggregation of tobacco mosaic virus (TMV) coat protein (CP) was studied. Judging from differential scanning calorimetry "melting" curves, TMV CP in the form of a trimer-pentamer mixture ("4S-protein") has very low thermal stability, with a transition temperature at about 40 degrees C. Thermally denatured TMV CP displayed high propensity for large (macroscopic) aggregate formation. TMV CP macroscopic aggregation was strongly dependent on the protein concentration and solution ionic strength. By varying phosphate buffer molarity, it was possible to merge or to separate the denaturation and aggregation processes. Using far-UV CD spectroscopy, it was found that on thermal denaturation TMV CP subunits are converted into an intermediate that retains about half of its initial alpha-helix content and possesses high heat stability. We suppose that this stable thermal denaturation intermediate is directly responsible for the formation of TMV CP macroscopic aggregates.

Panyukov Y. V., Nemykh M. A., Dobrov E. N. and Drachev V. A. (2007) 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.

Peters K., Hinz H. J., and Cesareni G. (1997) Introduction of a proline residue into position 31 of the loop of the dimeric 4-alpha-helical protein ROP causes a drastic destabilization. *Biol Chem* **378**, 1141-1152.

Abstract: The exchange of an alanine with a proline residue in position 31 of the loop region of the dimeric 4-alpha-helical-bundle protein ROP causes a reduction in the alpha-helix content of 7% and a reduction in stability of about 40% compared to the wild type parameters. The Gibbs energy of unfolding by denaturants extrapolated linearly to zero denaturant concentration, ΔG_0D (buffer, 25 degrees C), has

been determined to be 43 kJ (mol dimer)⁻¹. The corresponding ROPwt value is 72 kJ (mol dimer)⁻¹ (Steif et al., 1993). The extrapolated ΔG_0D values obtained from urea and GdmHCl un- and refolding studies are identical within error limits. Deconvolution of the stability values into enthalpy and entropy terms resulted in the following parameters. At $T_{1/2} = 43$ degrees C (Cprotein = 0.05 mg.ml⁻¹) the ROP A31P mutant is characterized by $\Delta H_v.H.0 = 272$ kJ (mol dimer)⁻¹, $\Delta C_p = 7.2$ kJ (mol dimer)⁻¹ K⁻¹, $\Delta S_0 = 762$ J (mol dimer)⁻¹ K⁻¹. These parameters are only approximately 50% as large as the corresponding values of ROPwt. We assume that the significant reduction in stability reflects the absence of at least one hydrogen bond as well as deformation of the protein structure. This interpretation is supported by the reduction in the change in heat capacity observed for the A31P mutant relative to ROPwt, by the increased aggregation tendency of the mutant and by the reduced specific CD absorption at 222 nm. All results support the view that in the case of ROP protein the loop region plays a significant role in the maintenance of native structure and conformational stability.

Photchanachai S., Mehta A., and Kitabatake N. (2002) Heating of an ovalbumin solution at neutral pH and high temperature. *Biosci Biotechnol Biochem* **66**, 1635-1640.

Abstract: The thermal denaturation, aggregation, and degradation of hen egg white ovalbumin dissolved in distilled and deionized water (60 mg/ml, pH 7.5) was investigated by differential scanning calorimetry (DSC), polyacrylamide gel electrophoresis (PAGE), and viscosity measurement. Two independent endothermic peaks were observed up to 180 degrees C by the DSC analysis. The first peak appeared at around 80 degrees C, corresponding to the denaturation temperature of ovalbumin. The second peak occurred around 140 degrees C due to the degradation of protein molecules as judged from the analysis by SDS-PAGE. The viscosity of the ovalbumin solution increased dramatically above 88 degrees C and maintained almost the same value up until heating to 140 degrees C. The increase in viscosity after heating to 88 degrees C was due to the denaturation and subsequent aggregation of ovalbumin molecules as observed by SDS-PAGE. The decrease in viscosity of the samples heated above 150 degrees C appears to have been the result of degradation of the ovalbumin molecules.

Pico G. A. (1997) Thermodynamic features of the thermal unfolding of human serum albumin. *Int J Biol Macromol* **20**, 63-73.

Abstract: The unfolding process of human serum albumin (HSA) was studied by thermal effect on the native fluorescence of the protein, thermal inactivation of the hydrolase activity of albumin and differential scanning calorimetry using the high sensitive calorimeter developed by Privalov. The denaturation process can be described by an approximation of the model of Eyring and Lumry: native [symbol: see text] unfolded reversible [symbol: see text] unfolded irreversible. It was found that the rate of irreversible step was very slow (at temperatures below 74 degrees C), allowing the resolution of the denaturation process as a reversible one on the basis of two states approximation. However, the presence of intramolecular cooperation in the thermal denaturation process at temperatures above 74 degrees C cannot be discarded, which might be favoring the aggregation of albumin molecules. The midpoint temperature of unfolding obtained by differential scanning calorimetry was of 63.1 degrees C +/- 0.4 at pH 7.4. This value was independent of the rate of scanning and it is in agreement with those obtained by techniques such as thermal effect on the protein fluorescence and on the hydrolase activity of albumin. The enthalpy of unfolding at pH 7.4 was 88.9 +/- 4 Kcal/mol. This value was low compared with those obtained for other proteins, suggesting the presence of a molten globule in the unfolding pathway of albumin. The neutral-basic conformational change (pH 7.4) of albumin did not modify the thermal stability and the enthalpy of denaturation of the protein. A pH below 4.3 (transition acid-neutral) the presence of a second peak in the thermogram of albumin with a T_M of 46.2 degrees C +/- 0.9 would be suggesting a loss of cooperativity between the various domains of albumin in the unfolding.

Pivovarova A. V., Mikhailova V. V., Chernik I. S., Chebotareva N. A., Levitsky D. I., and Gusev N. B. (2005) Effects of small heat shock proteins on the thermal denaturation and aggregation of F-actin. *Biochem Biophys Res Commun* **331**, 1548-1553.

Abstract: Effect of recombinant chicken small heat shock protein with molecular mass 24 kDa (Hsp24) and recombinant human small heat shock protein with molecular mass 27 kDa (Hsp27) on the heat-induced denaturation and aggregation of skeletal F-actin was analyzed by means of differential scanning calorimetry and light scattering. All small heat shock proteins did not affect thermal unfolding of F-actin measured by differential scanning calorimetry, but effectively prevented aggregation of thermally

denatured actin. Small heat shock protein formed stable complexes with denatured (but not with intact) F-actin. The size of these highly soluble complexes was smaller than the size of intact F-actin filaments. It is supposed that protective effect of small heat shock proteins on the cytoskeleton is at least partly due to prevention of aggregation of denatured actin.

Plaza d. P., Ibarra-Molero B., and Sanchez-Ruiz J. M. (2000) Lower kinetic limit to protein thermal stability: a proposal regarding protein stability in vivo and its relation with misfolding diseases. *Proteins* **40**, 58-70.

Abstract: In vitro thermal denaturation experiments suggest that, because of the possibility of irreversible alterations, thermodynamic stability (i.e., a positive value for the unfolding Gibbs energy) does not guarantee that a protein will remain in the native state during a given timescale. Furthermore, irreversible alterations are more likely to occur in vivo than in vitro because (a) some irreversible processes (e.g., aggregation, "undesirable" interactions with other macromolecular components, and proteolysis) are expected to be fast in the "crowded" cellular environment and (b) in many cases, the relevant timescale in vivo (probably related to the half-life for protein degradation) is expected to be longer than the timescale of the usual in vitro experiments (of the order of minutes). We propose, therefore, that many proteins (in particular, thermophilic proteins and "complex" proteins systems) are designed (by evolution) to have significant kinetic stability when confronted with the destabilizing effect of irreversible alterations. We show that, as long as these alterations occur mainly from non-native states (a Lumry-Eyring scenario), the required kinetic stability may be achieved through the design of a sufficiently high activation barrier for unfolding, which we define as the Gibbs energy barrier that separates the native state from the non-native ensemble (unfolded, partially folded, and misfolded states) in the following generalized Lumry-Eyring model: Native State \leftrightarrow Non-Native Ensemble \rightarrow Irreversibly Denatured Protein. Finally, using familial amyloid polyneuropathy (FAP) as an illustrative example, we discuss the relation between stability and amyloid fibril formation in terms of the above viewpoint, which leads us to the two following tentative suggestions: (a) the hot spot defined by the FAP-associated amyloidogenic mutations of transthyretin reflects the structure of the transition state for unfolding and (b) substances that decrease the in vitro rate of transthyretin unfolding could also be inhibitors of amyloid fibril formation.

Potekhin S. A., Melnik T. N., Popov V., Lanina N. F., Vazina A. A., Rigler P., Verdini A. S., Corradin G., and Kajava A. V. (2001) De novo design of fibrils made of short alpha-helical coiled coil peptides. *Chem Biol* **8**, 1025-1032.

Abstract: BACKGROUND: The alpha-helical coiled coil structures formed by 25-50 residues long peptides are recognized as one of Nature's favorite ways of creating an oligomerization motif. Known de novo designed and natural coiled coils use the lateral dimension for oligomerization but not the axial one. Previous attempts to design alpha-helical peptides with a potential for axial growth led to fibrous aggregates which have an unexpectedly big and irregular thickness. These facts encouraged us to design a coiled coil peptide which self-assembles into soluble oligomers with a fixed lateral dimension and whose alpha-helices associate in a staggered manner and trigger axial growth of the coiled coil. Designing the coiled coil with a large number of subunits, we also pursue the practical goal of obtaining a valuable scaffold for the construction of multivalent fusion proteins. RESULTS: The designed 34-residue peptide self-assembles into long fibrils at slightly acid pH and into spherical aggregates at neutral pH. The fibrillogenesis is completely reversible upon pH change. The fibrils were characterized using circular dichroism spectroscopy, sedimentation diffusion, electron microscopy, differential scanning calorimetry and X-ray fiber diffraction. The peptide was deliberately engineered to adopt the structure of a five-stranded coiled coil rope with adjacent alpha-helices, staggered along the fibril axis. As shown experimentally, the most likely structure matches the predicted five-stranded arrangement. CONCLUSIONS: The fact that the peptide assembles in an expected fibril arrangement demonstrates the credibility of our conception of design. The discovery of a short peptide with fibril-forming ability and stimulus-sensitive behavior opens new opportunities for a number of applications.

Pouvreau L., Kroef T., Gruppen H., van Koningsveld G., van den Broek L. A., and Voragen A. G. (2005) Structure and stability of the potato cysteine protease inhibitor group (cv. Elkana). *J Agric Food Chem* **53**, 5739-5746.

Abstract: The conformational stability of potato cysteine protease inhibitor (PCPI), the second most abundant protease inhibitor group in potato tuber, was investigated at ambient temperature and upon

heating using far- and near-UV circular dichroism spectroscopy, fluorescence spectroscopy, and differential scanning calorimetry (DSC). The PCPI isoforms investigated have a highly similar structure at both the secondary and the tertiary level. PCPI isoforms show structural properties similar to those of the potato serine protease inhibitor group and the Kunitz type soybean trypsin inhibitor, a known beta-II protein. Therefore, PCPI isoforms are also classified as members of the beta-II protein subclass. Results show that the thermal unfolding of PCPI isoforms does not follow a two-state mechanism and that at least one intermediate is present. The occurrence of this intermediate is most apparent in the thermal unfolding of PCPI 8.3 as indicated by the presence of two peaks in the DSC thermogram. Additionally, the formation of aggregates (>100 kDa), especially at low scan rates, increases the apparent cooperativity of the unfolding.

Pouvreau L., Gruppen H., van Koningsveld G., van den Broek L. A., and Voragen A. G. (2005) Conformational stability of the potato serine protease inhibitor group. *J Agric Food Chem* **53**, 3191-3196. **Abstract:** The thermal unfolding of potato serine protease inhibitor (PSPI), the most abundant protease inhibitor group in potato tuber, was measured using far UV CD spectroscopy, fluorescence spectroscopy, and DSC. The results indicate that the thermal as well as the guanidinium-induced unfolding of PSPI occurs via a non-two-state mechanism in which at least one stable intermediate is present. Additionally, the occurrence of aggregation, especially at low scan rates, increases the apparent cooperativity of the unfolding and makes the system kinetically rather than thermodynamically controlled. Aggregate formation seems to occur via a specific mechanism of which PSPI in a tetrameric form is the end product and which may involve disulfide interchanges.

Rakhit R., Crow J. P., Lepock J. R., Kondejewski L. H., Cashman N. R., and Chakrabartty A. (2004) Monomeric Cu,Zn-superoxide dismutase is a common misfolding intermediate in the oxidation models of sporadic and familial amyotrophic lateral sclerosis. *J Biol Chem* **279**, 15499-15504.

Abstract: Proteinacious intracellular aggregates in motor neurons are a key feature of both sporadic and familial amyotrophic lateral sclerosis (ALS). These inclusion bodies are often immunoreactive for Cu,Zn-superoxide dismutase (SOD1) and are implicated in the pathology of ALS. On the basis of this and a similar clinical presentation of symptoms in the familial (fALS) and sporadic forms of ALS, we sought to investigate the possibility that there exists a common disease-related aggregation pathway for fALS-associated mutant SODs and wild type SOD1. We have previously shown that oxidation of fALS-associated mutant SODs produces aggregates that have the same morphological, structural, and tinctorial features as those found in SOD1 inclusion bodies in ALS. Here, we show that oxidative damage of wild type SOD at physiological concentrations (approximately 40 μ M) results in destabilization and aggregation in vitro. Oxidation of either mutant or wild type SOD1 causes the enzyme to dissociate to monomers prior to aggregation. Only small changes in secondary and tertiary structure are associated with monomer formation. These results indicate a common aggregation prone monomeric intermediate for wild type and fALS-associated mutant SODs and provides a link between sporadic and familial ALS.

Remmele R. L., Jr., Zhang-van Enk J., Dharmavaram V., Balaban D., Durst M., Shoshitaishvili A., and Rand H. (2005) Scan-rate-dependent melting transitions of interleukin-1 receptor (type II): elucidation of meaningful thermodynamic and kinetic parameters of aggregation acquired from DSC simulations. *J Am Chem Soc* **127**, 8328-8339.

Abstract: The role of thermal unfolding as it pertains to thermodynamic properties of proteins and their stability has been the subject of study for more than 50 years. Moreover, exactly how the unfolding properties of a given protein system may influence the kinetics of aggregation has not been fully characterized. In the study of recombinant human Interleukin-1 receptor type II (rhuIL-1R(II)) aggregation, data obtained from size exclusion chromatography and differential scanning calorimetry (DSC) were used to model the thermodynamic and kinetic properties of irreversible denaturation. A break from linearity in the initial aggregation rates as a function of 1/T was observed in the vicinity of the melting transition temperature ($T(m)$) approximately 53.5 degrees C), suggesting significant involvement of protein unfolding in the reaction pathway. A scan-rate dependence in the DSC experiment testifies to the nonequilibrium influences of the aggregation process. A mechanistic model was developed to extract meaningful thermodynamic and kinetic parameters from an irreversibly denatured process. The model was used to simulate how unfolding properties could be used to predict aggregation rates at different temperatures above and below the $T(m)$ and to account for concentration dependence of reaction rates. The model was

shown to uniquely identify the thermodynamic parameters $\Delta C(P)$ (1.3 +/- 0.7 kcal/mol-K), $\Delta H(m)$ (74.3 +/- 6.8 kcal/mol), and $T(m)$ with reasonable variances.

Rezaei-Ghaleh N., Ebrahim-Habibi A., Moosavi-Movahedi A. A. and Nemat-Gorgani M. (2007) Effect of polyamines on the structure, thermal stability and 2,2,2-trifluoroethanol-induced aggregation of alpha-chymotrypsin. *Int J Biol Macromol.* **41**, 597-604.

Abstract: Naturally occurring polyamines are known to interact with a variety of biomolecules and critically involve in some important physiological processes. They have also been shown to influence protein aggregation in vitro in some cases. The aim of the present study was to investigate how polyamines may influence the structure and thermal stability of alpha-chymotrypsin and modulate alcohol-induced aggregation of this protein. Various techniques, including turbidity measurements, tensiometry, DSC, intrinsic fluorescence and far- and near-UV circular dichroism spectroscopy were used to examine the effect of putrescine and spermidine on alpha-chymotrypsin. While slight changes in the secondary and tertiary structure of the protein was observed, a clear stabilizing effect against its thermal unfolding was achieved. Moreover, the polyamines were found to inhibit TFE-induced aggregation at 32% TFE and promote formation of non-native alpha-helices in the protein structure. Based on the observed increase in surface tension induced by polyamines, it is suggested that their effects on enhancing thermal stability and alcohol-induced alpha-helices formation may be due to their kosmotropic properties.

Roberge M., Lewis R. N., Shareck F., Morosoli R., Kluepfel D., Dupont C., and McElhaney R. N. (2003) Differential scanning calorimetric, circular dichroism, and Fourier transform infrared spectroscopic characterization of the thermal unfolding of xylanase A from *Streptomyces lividans*. *Proteins* **50**, 341-354.

Abstract: The thermal unfolding of xylanase A from *Streptomyces lividans*, and of its isolated substrate binding and catalytic domains, was studied by differential scanning calorimetry and Fourier transform infrared and circular dichroism spectroscopy. Our calorimetric studies show that the thermal denaturation of the intact enzyme is a complex process consisting of two endothermic events centered near 57 and 64 degrees C and an exothermic event centered near 75 degrees C, all of which overlap slightly on the temperature scale. A comparison of the data obtained with the intact enzyme and isolated substrate binding and catalytic domains indicate that the lower- and higher-temperature endothermic events are attributable to the thermal unfolding of the xylan binding and catalytic domains, respectively, whereas the higher-temperature exothermic event arises from the aggregation and precipitation of the denatured catalytic domain. Moreover, the thermal unfolding of the two domains of the native enzyme are thermodynamically independent and differentially sensitive to pH. The unfolding of the substrate binding domain is a reversible two-state process and, under appropriate conditions, the refolding of this domain to its native conformation can occur. In contrast, the unfolding of the catalytic domain is a more complex process in which two subdomains unfold independently over a similar temperature range. Also, the unfolding of the catalytic domain leads to aggregation and precipitation, which effectively precludes the refolding of the protein to its native conformation. These observations are compatible with the results of our spectroscopic studies, which show that the catalytic and substrate binding domains of the enzyme are structurally dissimilar and that their native conformations are unaffected by their association in the intact enzyme. Thus, the calorimetric and spectroscopic data demonstrate that the *S. lividans* xylanase A consists of structurally dissimilar catalytic and substrate binding domains that, although covalently linked, undergo essentially independent thermal denaturation. These observations provide valuable new insights into the structure and thermal stability of this enzyme and should assist our efforts at engineering xylanases that are more thermally robust and otherwise better suited for industrial applications.

Rochu D., Beaufet N., Renault F., Viguie N., and Masson P. (2002) The wild type bacterial Co(2+)/Co(2+)-phosphotriesterase shows a middle-range thermostability. *Biochim Biophys Acta* **1594**, 207-218.

Abstract: The phosphotriesterase (PTE) from *Pseudomonas diminuta*, a metalloenzyme that catalyses the hydrolysis of organophosphorus pesticides and nerve agents, has been described as a remarkably heat-stable protein [Grimsley et al., *Biochemistry* 36 (1997), 14366-14374]. Because substitution of the naturally occurring zinc ions by cobalt ions was found to enhance the enzyme catalytic activity, we investigated the thermal stability of the Co(2+)/Co(2+)-PTE. This study, carried out using capillary electrophoresis under optimised conditions in the pH range 9-10 compatible with optimal enzyme activity, provided evidence for irreversible denaturation according to the Lumry-Eyring model. A temperature-

induced conformational transition (T_m approximately equal to 58 degrees C) and an early growing of aggregates were observed. Comparison of UV spectra with heat-induced inactivation data clearly demonstrated that the PTE state populated above T_m was neither native nor active. Differential scanning calorimetry showed only an exothermic trace due to aggregation of the denatured protein at $T=76$ degrees C. Accordingly, the temperature-induced denaturation process of the PTE could be described by a consecutive reaction model, including formation of an intermediate with enhanced activity at T approximately equal to 45 degrees C and an inactive unfolded state populated at T approximately equal to 58 degrees C, which leads to denatured aggregates. Thus, the wild type Co(2+)/Co(2+)-PTE displays a middle-range thermostability. Hence, for decontamination purposes under extreme Earth temperatures, wild type and engineered mutants of PTE substituted with other metal cations should be evaluated.

Rojo-Dominguez A., Hernandez-Arana A., Mendoza-Hernandez G., and Rendon J. L. (1997) Thermal denaturation of glutathione reductase from cyanobacterium *Spirulina maxima*. *Biochem Mol Biol Int* **42**, 631-639.

Abstract: The thermal unfolding of glutathione reductase (NAD[P]H:GSSG oxidoreductase EC 1.6.4.2.) from cyanobacterium *Spirulina maxima* was monitored by differential scanning calorimetry and circular dichroism at neutral pH. Covalent cross-linking of enzyme at different temperatures revealed dimer as the species undergoing the thermal transition. A single endotherm was observed, but its thermodynamic parameters showed dependence on the scan rate. In the transition zone, aggregation of the dimeric species was observed. Analysis of the enzyme heated at 80 degrees C revealed that the resultant species retained a high content of secondary structure. The addition of low concentrations of guanidinium hydrochloride resulted in a full cooperative thermal transition. A model in which the dimeric protein undergoes a partial unfolding in a kinetically controlled fashion is proposed, such that the experimental value of $\Delta H(\text{cal})$ results from the simultaneous occurrence of endothermic and exothermic events.

Runser S. and Cerletti N. (1995) Transforming growth factors beta: conformational stability and features of the denaturation of recombinant human transforming growth factors beta 2 and beta 3. *Biotechnol Appl Biochem* **22 (Pt 1)**, 39-53.

Abstract: Transforming growth factors beta (TGF-beta) are cytokines with multiple biological activities. Their development as biopharmaceutical drugs targets the control of complex physiological processes such as osteogenesis and epithelial cell differentiation. We report here the first characterization of the recombinant human (rh) TGF-beta 2 and rhTGF-beta 3 isoforms in terms of their conformational stability and structural transitions induced by a chaotrope or temperature. The transitions detected by CD spectroscopy suggested that thermal denaturation of both TGF-beta isoforms apparently fitted a simple two-state ($N \rightleftharpoons D$) model. However, the ratios of calorimetric to van't Hoff enthalpies, significantly different from unity, indicated that these molecules most probably consist of independently denaturing subdomains. The complex transitions induced by guanidine hydrochloride, at pH 1.8 or 8.0, also suggested intermediately denatured structures. Thermodynamic stabilities under pH conditions useful for bioprocessing were derived from spectroscopic and calorimetric measurements. Treatment of thermal denaturation data by van't Hoff analysis yielded, for the beta 2 and beta 3 isoforms respectively, apparent $\Delta G(25 \text{ degrees C, pH 1.8})$ of 20.4/17.2 kJ/mol and 17.5/18.6 kJ/mol (near-UV CD/far-UV CD data) in 20 mM hydrochloric acid, and apparent $\Delta G(25 \text{ degrees C, pH 3.0})$ of 35.1 and 33.5 kJ/mol in 0.25 M acetic acid (calorimetric data). Neither low-pH-induced denatured states nor soluble aggregates were detected in both acidic solvents. The spectroscopic and thermodynamic data should be useful for assessing the homogeneity and proper folding of these recombinant molecules.

Sandberg A., Leckner J., Shi Y., Schwarz F. P., and Karlsson B. G. (2002) Effects of metal ligation and oxygen on the reversibility of the thermal denaturation of *Pseudomonas aeruginosa* azurin. *Biochemistry* **41**, 1060-1069.

Abstract: Thermodynamic equilibrium transition models in DSC are only applicable to reversible processes, but reversibility of the thermal transitions of proteins is comparatively rare because of intermolecular aggregation of denatured proteins and the degradation that occurs at high temperatures. The cupredoxin azurin from *Pseudomonas aeruginosa* has previously been found to exhibit irreversible thermal denaturation, both as holo- and apoprotein [Engeseth, H. R., and McMillin, D. R. (1986) *Biochemistry* **25**, 2448-2455]. In this study, however, we demonstrate that this beta-barrel protein of Greek key topology in fact unfolds reversibly in anaerobic solutions when nonreducible metal ions are ligated to the protein. We

show that it is the metal-coordinating cysteine residue (C112) that becomes exclusively oxidized in a transition metal catalyzed oxidation reaction with dissolved O₂ at high temperatures. Both Cu(I)- and Zn(II)-coordinating wild-type azurin therefore unfold reversibly in anaerobic solutions, as well as the Zn(II)-coordinating disulfide-deficient C3A/C26A mutant. Correspondingly, apoazurin mutants C112A and C112S unfold reversibly, even in aerobic solutions, and exhibit nearly perfect two-state transitions. Unfolding of Cu(II)-coordinating azurin is, on the other hand, always irreversible due to autoxidation of the thiolate resulting in Cu(I) and a thiyl radical prone to oxidation.

Sasahara K., Yagi H., Naiki H. and Goto Y. (2007) Heat-induced conversion of beta(2)-microglobulin and hen egg-white lysozyme into amyloid fibrils. *J Mol Biol* **372**, 981-991.

Abstract: Thermodynamic parameters characterizing protein stability can be obtained for a fully reversible folding/unfolding system directly by differential scanning calorimetry (DSC). However, the reversible DSC profile can be altered by an irreversible step causing aggregation. Here, to obtain insight into amyloid fibrils, ordered and fibrillar aggregates responsible for various amyloidoses, we studied the effects on human beta(2)-microglobulin and hen egg-white lysozyme of a combination of agitation and heating. Aggregates formed by mildly agitating protein solutions in the native state in the presence of NaCl were heated in the cell of the DSC instrument. For beta(2)-microglobulin, with an increase in the concentration of NaCl at neutral pH, the thermogram began to show an exothermic transition accompanied by a large decrease in heat capacity, followed by a kinetically controlled thermal response. Similarly, the aggregated lysozyme at a high concentration of NaCl revealed a similar distinct transition in the DSC thermogram over a wide pH range. Electron microscopy demonstrated the conformational change into amyloid fibrils. Taken together, the combined use of agitation and heating is a powerful way to generate amyloid fibrils from two proteins, beta(2)-microglobulin and hen egg-white lysozyme, and to evaluate the effects of heat on fibrillation, in which the heat capacity is crucial to characterizing the transition.

Satish K. M., Mrudula T., Mitra N., and Bhanuprakash R. G. (2004) Enhanced degradation and decreased stability of eye lens alpha-crystallin upon methylglyoxal modification. *Exp Eye Res* **79**, 577-583.

Abstract: Methylglyoxal (MGO), a potent glycation agent, forms advanced glycation end products (AGEs) with proteins. Several diabetic complications including cataract are thought to be the result of accumulation of these protein-AGEs. alpha-Crystallin, molecular chaperone of the eye lens, plays an important role in maintaining the transparency of the lens by preventing the aggregation/inactivation of several proteins/enzymes in addition to its structural role. Binding of adenosine-5-triphosphate (ATP) to alpha-crystallin has been shown to enhance its chaperone-like function and protection against proteolytic degradation. In the earlier study, we have shown that modification of alpha-crystallin by MGO caused altered chaperone-like activity along with structural changes, cross-linking, coloration and subsequent insolubilization leading to scattering of light [Biochem. J. 379 (2004) 273]. In the present study, we have investigated ATP binding, stability and degradation of MGO-modified alpha-crystallin. Proteolytic digestion with trypsin and chymotrypsin showed that MGO-modified alpha-crystallin is more susceptible to degradation compared to native alpha-crystallin. Furthermore, ATP was able to protect native alpha-crystallin against proteolytic cleavage but not MGO-modified alpha-crystallin. Interestingly, binding studies indicate decreased ATP binding to MGO-modified alpha-crystallin and support the decreased protection by ATP against proteolysis. In addition, differential scanning calorimetric and denaturant-induced unfolding studies indicate that modification of alpha-crystallin by MGO leads to decreased stability. These results indicate that MGO-modification of alpha-crystallin causes partial unfolding and decreased stability leading to enhanced proteolysis. Cross-linking of these degraded products could result in aggregation and subsequent insolubilization as observed in senile and diabetic cataract lenses.

Schwartz D., Sofia S., and Friess W. (2006) Integrity and stability studies of precipitated rhBMP-2 microparticles with a focus on ATR-FTIR measurements. *Eur J Pharm Biopharm* **63**, 241-248.

Abstract: A major obstacle in the development of protein drug formulations is the need to maintain the native, active protein structure both during the formulation process and upon long time storage. Controlled precipitation was evaluated for its potential to supply stable microparticulate formulations of bone-regenerating recombinant human Bone Morphogenetic Protein-2 (rhBMP-2). Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) did provide insight into the protein formulation and stability. Temperature dependent ATR-FTIR measurements and DSC measurements allow for the study of changes in the protein structure during melting. To address the question of isomerization, peptide mapping

was performed, and protein aggregation was monitored by size exclusion chromatography (SEC). It could be demonstrated by ATR-FTIR that controlled precipitation did not harm the protein and the process is fully reversible. DSC measurements further confirmed these findings. No changes in the transition temperature and process were observed after precipitation and redissolution. Upon storage, isomerization and aggregation could be detected, but to a lower extent in the precipitated formulation as compared to a solution reference. Thus, controlled precipitation of rhBMP-2 is fully reversible and has the potential as alternative formulation tool for the generation of a microparticulate drug delivery system.

Shiraki K., Kudou M., Nishikori S., Kitagawa H., Imanaka T., and Takagi M. (2004) Arginine ethylester prevents thermal inactivation and aggregation of lysozyme. *Eur J Biochem* **271**, 3242-3247.

Abstract: Arginine is a versatile additive to prevent protein aggregation. This paper shows that arginine ethylester (ArgEE) prevents heat-induced inactivation and aggregation of hen egg lysozyme more effectively than arginine or guanidine. The addition of ArgEE decreased the melting temperature of lysozyme. This data could be interpreted in terms of ArgEE binding to unfolded lysozyme, possibly through the ethylated carboxyl group, which leads to effective prevention of intermolecular interaction among aggregation-prone molecules. The data suggest that ArgEE could be used as an additive to prevent inactivation and aggregation of heat-labile proteins.

Shalova I.N., Asryants R.A., Sholukh M.V., Saso L., Kurganov B.I., Muronetz V.I., and Izumrudov V.A. (2005) Interaction of polyanions with basic proteins, 2(a) : influence of complexing polyanions on the thermo-aggregation of oligomeric enzymes. *Macromol Biosci.* **5**, 1184-92.

Abstract: The ability of synthetic polyanions to suppress thermo-aggregation of the oligomeric enzymes (glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, and aspartate aminotransferase) has been established. The ability of the polyanions to reduce the thermo-aggregation increased in the order poly(methacrylic acid) < poly(acrylic acid) < sodium poly(styrene sulphonate), which agreed well with the increase, in the same order, of the charge density of the chains. The lengthening of the chains, as well as the rise in their relative content, resulted in an increase of the ability to reduce thermo-aggregation, mentioned above. Complete prevention of the enzyme aggregation was achieved when highly charged polyanions of a relatively high degree of polymerization were used in a concentration sufficient to solubilize the protein. Complexing with the polyanions prevented thermo-aggregation of the enzymes, but not their thermo-denaturation. The adverse effect of the complexing polyanions on the catalytic activity was reduced by the addition of a synthetic polycation, which resulted in a significant reactivation (up to 40%) of the enzyme. The possibility of preventing the thermo-aggregation of enzyme molecules and then partly restoring the enzyme activity, appears to be of particular interest when studying the aggregation mechanism of proteins that are prone to form the amyloid structures responsible for the development of neurodegenerative diseases like Alzheimer's disease, bovine spongiform encephalopathy and Huntington disease. This finding can also be considered as an important step in the creation of artificial chaperones.

Sharma V. K. and Kalonia D. S. (2003) Temperature- and pH-induced multiple partially unfolded states of recombinant human interferon-alpha2a: possible implications in protein stability. *Pharm Res* **20**, 1721-1729.

Abstract: PURPOSE: To study the effect of solution conditions on the structural conformation of recombinant human interferon-alpha2a (IFNalpha2a) to investigate its tendency to form partially unfolded intermediates. METHODS: The structural properties of IFNalpha2a were studied at various pH values (2.0-7.4) and temperatures (5 degrees C-80 degrees C) using Trp fluorescence emission, fluorescence quenching, near- and far-UV circular dichroism (CD) spectroscopy, and DSC. RESULTS: Fluorescence intensity measurements as a function of temperature indicated the onset of the thermal unfolding of IFNalpha2a, denoted by Td, around 60 degrees C above pH 4.0. Td was not observed at pH 3.5 and below. Acrylamide and iodide quenching studies indicated partial unfolding of protein with decrease in pH and with increase in temperature up to 50 degrees C. Near-UV CD studies indicated a significant loss in the tertiary structure of protein on increase in temperature from 15 degrees C to 50 degrees C at all solution pHs. DSC scans supported results obtained from fluorescence and CD studies at pH 4.0 and below. DSC, however, was insensitive to changes that occurred at moderate temperatures at pH 5.0 and 7.4. CONCLUSIONS: IFNalpha2a has a tendency to acquire multiple partially unfolded states with structural conformations sensitive to solution pH and temperature. These states were formed at moderate

temperatures, and it is speculated that these partially unfolded states could play an important role in the aggregation of proteins during the long-term storage of aqueous protein formulations.

Shiraki K., Kudou M., Nishikori S., Kitagawa H., Imanaka T., and Takagi M. (2004) Arginine ethylester prevents thermal inactivation and aggregation of lysozyme. *Eur J Biochem* **271**, 3242-3247.

Abstract: Arginine is a versatile additive to prevent protein aggregation. This paper shows that arginine ethylester (ArgEE) prevents heat-induced inactivation and aggregation of hen egg lysozyme more effectively than arginine or guanidine. The addition of ArgEE decreased the melting temperature of lysozyme. This data could be interpreted in terms of ArgEE binding to unfolded lysozyme, possibly through the ethylated carboxyl group, which leads to effective prevention of intermolecular interaction among aggregation-prone molecules. The data suggest that ArgEE could be used as an additive to prevent inactivation and aggregation of heat-labile proteins.

Shiraki K., Kudou M., Fujiwara S., Imanaka T., and Takagi M. (2002) Biophysical effect of amino acids on the prevention of protein aggregation. *J Biochem (Tokyo)* **132**, 591-595.

Abstract: Each protein folds into a unique and native structure spontaneously. However, during the unfolding or refolding process, a protein often tends to form aggregates. To establish a method to prevent undesirable protein aggregation and to increase the stability of native protein structures under deterioration conditions, two types of aggregation conditions, thermal unfolding-induced aggregation and dilution-induced aggregation from denatured state, were studied in the presence of additional amino acids and ions using lysozyme as a model protein. Among 15 amino acids tested, arginine exhibited the best results in preventing the formation of aggregates in both cases. Further biophysical studies revealed that arginine did not change the thermal denaturation temperature (T_m) of the lysozyme. The preventive effect of arginine on aggregation was not dependent on the size or isoelectric point of eight kinds of proteins tested.

Shukla A., Raje M., and Guptasarma P. (2003) A backbone-reversed form of an all-beta alpha-crystallin domain from a small heat-shock protein (retro-HSP12.6) folds and assembles into structured multimers. *J Biol Chem* **278**, 26505-26510.

Abstract: The structural consequences of polypeptide backbone reversal ("retro" modification) remain largely unexplored, in particular, for the retro forms of globular all-beta-sheet proteins. To examine whether the backbone-reversed form of a model all-beta-sheet protein can fold and adopt secondary and tertiary structure, we created and examined the recombinant retro form of a 110-residue-long polypeptide, an alpha-crystallin-like small heat-shock protein, HSP12.6, from *C. elegans*. Following intracellular overexpression in fusion with a histidine affinity tag in *Escherichia coli*, purification under denaturing conditions, and removal of denaturant through dialysis, retro-HSP12.6 was found to fold to a soluble state. The folded protein was examined using fluorescence and CD spectroscopy, gel filtration chromatography, non-denaturing electrophoresis, differential scanning calorimetry, and electron microscopy and confirmed to have adopted secondary structure and assembled into a multimer. Interestingly, like its parent polypeptide, retro-HSP12.6 did not aggregate upon heating; rather, heating led to a dramatic increase in structural content and the adoption of what would appear to be a very well folded state at high temperatures. However, this was essentially reversed upon cooling with some hysteresis being observed resulting in greater structural content in the heated-cooled protein than in the unheated protein. The heated-cooled samples displayed CD spectra indicative of structural content comparable to that of any naturally occurring globular protein. Attempts are being made to refine crystallization conditions for the folded protein.

Singh N., Liu Z., and Fisher H. F. (1996) The existence of a hexameric intermediate with molten-globule-like properties in the thermal denaturation of bovine-liver glutamate dehydrogenase. *Biophys Chem* **63**, 27-36.

Abstract: We have studied the thermal denaturation of hexameric beef-liver glutamate dehydrogenase by itself and in the presence of ADP and guanidine-HCl by a variety of techniques. In differential scanning calorimetry studies, the observed melting temperature and total enthalpy of denaturation show no dependence on protein concentration, but do show significant dependence on the scan rate. This suggests that the overall denaturation process is irreversible and kinetically controlled. Isothermal unfolding kinetics from spectrophotometry confirm this result. The size of the protein, as shown by quasi-elastic light scattering measurements, does not change during the denaturation process. We interpret these results in

terms of the following model: N6 reversible N'6 \leftrightarrow 6U \leftrightarrow F) where N6 and N'6 are, respectively, the native hexamer and a hexameric, highly folded high-enthalpy species, U is the unfolded monomer and F is some final aggregated state. The kinetic intermediate, N'6, possesses the properties of one definition of a molten globule, having a very high enthalpy and a hexameric compact structured form. This "molten globule" is an obligatory intermediate in the unfolding pathway of the protein. The stabilization of the protein by ADP is due to the modulation of the high-enthalpy two-state pre-denaturation E reversible E' transition, resulting in the lowering of the energy of the native state of the protein.

Singh S. and Singh J. (2003) Effect of polyols on the conformational stability and biological activity of a model protein lysozyme. *AAPS PharmSciTech* **4**, E42.

Abstract: The purpose of this study was to investigate the stabilizing action of polyols against various protein degradation mechanisms (eg, aggregation, deamidation, oxidation), using a model protein lysozyme. Differential scanning calorimeter (DSC) was used to measure the thermodynamic parameters, mid point transition temperature and calorimetric enthalpy, in order to evaluate conformational stability. Enzyme activity assay was used to corroborate the DSC results. Mannitol, sucrose, lactose, glycerol, and propylene glycol were used as polyols to stabilize lysozyme against aggregation, deamidation, and oxidation. Mannitol was found to stabilize lysozyme against aggregation, sucrose against deamidation both at neutral pH and at acidic pH, and lactose against oxidation. Stabilizers that provided greater conformational stability of lysozyme against various degradation mechanisms also protected specific enzyme activity to a greater extent. It was concluded that DSC and bioassay could be valuable tools for screening stabilizers in protein formulations.

Smirnovas V. and Winter R. (2008) Revealing different aggregation pathways of amyloidogenic proteins by ultrasound velocimetry. *Biophys J* **94**, 3241-3246.

Abstract: In this work, we performed a detailed thermodynamic study, including ultrasound velocimetry, densimetry, calorimetry, and FTIR spectroscopy, of an aggregation-prone protein (insulin) under different salt-screening conditions to gain a deeper insight into the scenario of physicochemical events during its temperature-induced unfolding and aggregation reactions. Differences in aggregation and fibrillization pathways are reflected in changes of the partial molar volume, the coefficients of thermal expansion and compressibility, and the infrared spectral properties of the protein. Combining all experimental data allows setting up a scheme for the temperature-dependent insulin aggregation reaction in the presence and absence of NaCl. As revealed by complementary atomic force microscopy studies, under charge-screening conditions, a process involving structural reorganization, ripening, and formation of more compact nuclei from amorphous oligomers is involved in the formation of mature fibrillar morphologies. In this work, our focus was to put forward a comprehensive discussion of the use of ultrasound velocimetry in disentangling different aggregation pathways. In fact, ultrasound velocimetry proved to be very sensitive to changes in aggregation pathway, highlighting the importance of density and compressibility changes in the different aggregation and fibrillization reactions of the protein

Snyder S. W., Edalji R. P., Lindh F. G., Walter K. A., Solomon L., Pratt S., Steffy K., and Holzman T. F. (1996) Initial characterization of autoprocessing and active-center mutants of CMV proteinase. *J Protein Chem* **15**, 763-774.

Abstract: Human cytomegalovirus (CMV) encodes a unique serine proteinase that is required in the maturation of the viral capsid. The CMV proteinase can undergo autocatalytic activation and is subject to proteolytic self-inactivation. Mutant enzyme forms were prepared to eliminate the initial autoprocessing site and thus form an active single-chain protein for structure-function studies. Two mutants of CMV proteinase were cloned and expressed in *Escherichia coli*. The A143V mutant was a conservative substitution at the first internal cleavage site. The S132A mutant modified one of the triad of residues responsible for catalytic activity. Through the use of computer-controlled high-cell-density fermentations the mutant proteins were expressed in *E. coli* at approximately 170 mg/L as both soluble (approximately 40% of total) and inclusion-body forms (approximately 60% of total). The soluble enzyme was purified by standard methods; inclusion-body protein was isolated by standard methods after refolding and solubilization in guanidine or urea. Sedimentation equilibrium and sedimentation velocity analyses reveal that the enzyme undergoes concentration-dependent aggregation. It exhibits a monomer \rightleftharpoons dimer equilibrium ($K_d = 1 \mu\text{M}$) at low concentrations and remains dimeric at high concentrations (28 mg/ml). Differential scanning calorimetry data for protein thermal unfolding fit best to a non-two-state model with

two components ($T_m = 52.3$ and 55.3 degrees C) which subsequently aggregate upon unfolding. Analysis of the short-UV circular dichroism spectra of protein forms resulting from expression as soluble molecules (not refolded) reveals that the two mutants have very similar secondary structures which comprise a mixed structural motif of 20% alpha-helix, 26% beta-sheet, and 53% random coil. Though soluble and active (A143V mutant only), CD analysis revealed that protein refolded from inclusion bodies did not exhibit spectra identical to that of protein expressed only in soluble form.

Stathopoulos P. B., Rumfeldt J. A., Karbassi F., Siddall C. A., Lepock J. R., and Meiering E. M. (2006) Calorimetric analysis of thermodynamic stability and aggregation for apo and holo amyotrophic lateral sclerosis-associated Gly-93 mutants of superoxide dismutase. *J Biol Chem* **281**, 6184-6193.

Abstract: Differential scanning calorimetry was used to measure changes in thermodynamic stability and aggregation for glycine 93 mutants of human copper, zinc-superoxide dismutase (SOD). Glycine 93 is a conserved residue at position $i + 3$ of a tight turn and has been found to be a mutational hot spot in familial amyotrophic lateral sclerosis (fALS). The fALS-associated mutations, G93A, G93S, G93R, G93D, and G93V, were made in a pseudo wild-type background containing no free cysteines, which prevented the formation of aberrant disulfide bonds upon thermal unfolding, and enabled quantitative thermodynamic analysis of the effects of the mutations. Thermal unfolding was highly reversible for all the SODs in both the fully metallated (holo) and metal-free (apo) forms. The data for all the holo-SODs and for the apo-pseudo-wild-type SOD were well fit by a 2-state unfolding model for native dimer (N2) to two unfolded monomers (2U), $N2 \leftrightarrow 2U$. The holo- and apo-forms of the mutants are significantly destabilized (by 1.5-3.5 kcal mol⁻¹ monomer) relative to the corresponding forms of pseudo wild-type, with the relative stabilities being correlated with statistical preferences for amino acids in this structural context. Although van't Hoff (ΔH_{vH}) to calorimetric (ΔH_{cal}) enthalpy ratios are close to unity for all the holo-SODs and for apo-pseudo-wild-type, consistent with a 2-state transition, ΔH_{vH} is considerably larger than ΔH_{cal} for all the apo-mutants. This suggests that the mutations cause apo-SOD to have an increased propensity to misfold or aggregate, which may be linked to increased toxic mutant SOD aggregation in fALS.

Stefanini S., Cavallo S., Wang C. Q., Tataseo P., Vecchini P., Giartosio A., and Chiancone E. (1996) Thermal stability of horse spleen apoferritin and human recombinant H apoferritin. *Arch Biochem Biophys* **325**, 58-64.

Abstract: The thermal stability of horse spleen apoferritin, a heteropolymer composed of 90% L and 10% H chains, has been studied by differential scanning calorimetry and compared with that of the human recombinant H homopolymer. The denaturation temperatures (T_m) are significantly higher for the horse spleen polymer than for the recombinant protein under all experimental conditions (e.g., at pH 7, T_m values are $>$ or $= 93$ and 77 degrees C, respectively). The thermal denaturation process displays substantial reversibility for both polymers up to a few degrees below T_m , as indicated by CD measurements in the far and near uv regions. At temperatures higher than T_m the thermograms are influenced by the exothermic contribution of aggregation and/or precipitation. The H homopolymer thermogram, which is not distorted by the exotherm, is consistent with a multistate denaturation process. Acid dissociation of apoferritin produces stable dimeric subunits. The thermal unfolding of both dimeric subunits is reversible at least up to T_m and is characterized by an inversion of stability relative to the polymers (at pH 3.5, T_m is 42 degrees C for the horse spleen and 50 degrees C for the H subunit). These results indicate that the stabilization of the polymeric structure arises mainly from interactions between dimers, in accordance with the crystallographic evidence that the dimers are the building blocks of the polymeric molecule.

Stirpe A., Rizzuti B., Pantusa M., Bartucci R., Sportelli L. and Guzzi R. (2008) Thermally induced denaturation and aggregation of BLG-A: effect of the Cu(2+) and Zn (2+) metal ions. *Eur Biophys J* **37**, 1351-1360.

Abstract: There is growing evidence that metal ions can accelerate the aggregation process of several proteins. This process, associated with several neuro-degenerative diseases, has been reported also for non-pathological proteins. In the present work, the effects of copper and zinc ions on the denaturation and aggregation processes of beta-lactoglobulin A (BLG-A) are investigated by differential scanning calorimetry (DSC), fluorescence, electron paramagnetic resonance (EPR) and optical density. The DSC profiles reveal that the thermal behaviour of BLG-A is a complex process, strongly dependent on the protein concentration. For concentrations ≤ 0.13 mM, the thermogram shows an endothermic peak at 84.3

degrees C, corresponding to denaturation; for concentrations >0.13 mM an exothermic peak also appears, above 90 degrees C, related to the aggregation of the denaturated BLG-A molecules. The thioflavin T fluorescence indicates that the thermally induced aggregates show fibrillar features. The presence of either equimolar Cu(2+) or Zn(2+) ions in the protein solution has different effects. In particular, copper binds to the protein in the native state, as evidenced by EPR experiments, and destabilizes BLG-A by decreasing the denaturation temperature by about 10 degrees C, whereas zinc ions probably perturb the partially denaturated state of the protein. The kinetics of BLG-A aggregation shows that both metal ions abolish the lag phase before the aggregation starts. Moreover, the rate of the process is 4.6-fold higher in the presence of copper, whereas the effect of zinc is negligible. The increase of the aggregation rate, induced by copper, may be due to a site-specific binding of the metal ion on the protein

Sudharshan E. and Rao A. G. (1999) Involvement of cysteine residues and domain interactions in the reversible unfolding of lipoxygenase-1. *J Biol Chem* **274**, 35351-35358.

Abstract: Urea-induced unfolding of lipoxygenase-1 (LOX1) at pH 7.0 was followed by enzyme activity, spectroscopic measurements, and limited proteolysis experiments. Complete unfolding of LOX1 in 9 M urea in the presence of thiol reducing or thiol modifying reagents was observed. The aggregation and oxidative reactions prevented the reversible unfolding of the molecule. The loss of enzyme activity was much earlier than the structural loss of the molecule during the course of unfolding, with the midpoint concentrations being 4.5 and 7.0 M for activity and spectroscopic measurements, respectively. The equilibrium unfolding transition could be adequately fitted to a three-state, two-step model (N left arrow over right arrow I left arrow over right arrow U) and the intermediate fraction was maximally populated at 6.3 M urea. The free energy change ($\Delta G(H(2)O)$) for the unfolding of native (N) to intermediate (I) was 14.2 +/- 0.28 kcal/mol and for the intermediate to the unfolded state (U) was 11.9 +/- 0.12 kcal/mol. The ANS binding measurements as a function of urea concentration indicated that the maximum binding of ANS was in 6.3 M urea due to the exposure of hydrophobic groups; this intermediate showed significant amount of tertiary structure and retained nearly 60% of secondary structure. The limited proteolysis measurements showed that the initiation of unfolding was from the C-terminal domain. Thus, the stable intermediate observed could be the C-terminal domain unfolded with exposed hydrophobic domain-domain interface. Limited proteolysis experiments during refolding process suggested that the intermediate refolded prior to completely unfolded LOX1. These results confirmed the role of cysteine residues and domain-domain interactions in the reversible unfolding of LOX1. This is the first report of the reversible unfolding of a very large monomeric, multi-domain protein, which also has a prosthetic group.

Takahashi N., Tatsumi E., Orita T., and Hirose M. (1996) Role of the intrachain disulfide bond of ovalbumin during conversion into S-ovalbumin. *Biosci Biotechnol Biochem* **60**, 1464-1468.

Abstract: Disulfide-reduced and carboxymethylated ovalbumin was treated at pH 9.9 and 55 degrees C for 24 h as a specific condition for preparation of S-ovalbumin. The stability and conformation of the product were investigated. Such alkaline treatment converted native protein to S-ovalbumin, but this modified ovalbumin was not stabilized, according to results of calorimetric analysis. Instead, it had lost its native like conformation; the magnitude of CD spectra decreased. The conformation after alkaline treatment was not clear, but the possibility of aggregation was excluded by electrophoretic analysis. These observations showed that the transformation of native ovalbumin into S-ovalbumin requires the presence of the disulfide bond.

Tandang M. R., Adachi M., Inui N., Maruyama N., and Utsumi S. (2004) Effects of protein engineering of canola procruciferin on its physicochemical and functional properties. *J Agric Food Chem* **52**, 6810-6817.

Abstract: The primary structure of Brassica napus procruciferin 2/3a was engineered to elucidate structure-function relationships and to improve the functionality of cruciferin. The following mutants were constructed: (1) C287T, (2) DeltaII, variable region II was deleted; (3) C287T/DeltaII, mutation involving (1) and (2); (4) DeltaIV + A1aIV; and (5) DeltaIV + A3IV, variable region IV was replaced with variable region IV containing many charged residues from soybean glycinin A1aB1b and A3B4 subunits. Differential scanning calorimetry analysis revealed that the A1aIV region has a more favorable interaction with the procruciferin molecule than does A3IV as well as the original regions. On the basis of heat-induced precipitation analysis, it was concluded that replacement of the free cysteine residue with threonine (C287T) and insertion of charged regions (DeltaIV + A1aIV and DeltaIV + A3IV) could lead procruciferin to form soluble aggregates after heating. Low solubility was observed in mutants DeltaIV + A3IV, DeltaII,

and C287T/DeltaII, especially between pH 4 and 6 at $\mu = 0.08$, but not in DeltaIV + A1aIV, indicating that the number of acidic amino acid residues and the high number of glutamine residues are important factors for solubility at $\mu = 0.08$. None of the mutants showed any improvements in emulsifying ability, indicating that destabilization and addition of the hydrophilic region are not effective for emulsification. The insertion of the A1aIV region in procruciferin made the molecule more susceptible to alpha-chymotrypsin.

Tang C. H., Ten Z., Wang X. S., and Yang X. Q. (2006) Physicochemical and functional properties of hemp (*Cannabis sativa* L.) protein isolate. *J Agric Food Chem* **54**, 8945-8950.

Abstract: The amino acid composition and physicochemical and functional properties of hemp (*Cannabis sativa* L.) protein isolate (HPI) were evaluated and compared with those of soy protein isolate (SPI). Edestin, a kind of hexameric legumin, was the major protein component. HPI had similar or higher levels of essential amino acids (except lysine), in comparison to those amino acids of SPI. The essential amino acids in HPI (except lysine and sulfur-containing amino acids) are sufficient for the FAO/WHO suggested requirements for 2-5 year old children. The protein solubility (PS) of HPI was lower than that of SPI at pH less than 8.0 but similar at above pH 8.0. HPI contained much higher free sulfhydryl (SH) content than SPI. Differential scanning calorimetry analysis showed that HPI had only one endothermic peak with denaturation temperature (T(d)) of about 95.0 degrees C, attributed to the edestin component. The T(d) of the endotherm was nearly unaffected by 20-40 mM sodium dodecyl sulfate but significantly decreased by 20 mM dithiothreitol ($P < 0.05$). The emulsifying activity index, emulsion stability index, and water-holding capacity of HPI were much lower than those of SPI, and the fat adsorption capacity was similar. The data suggest that HPI can be used as a valuable source of nutrition for infants and children but has poor functional properties when compared with SPI. The poor functional properties of HPI have been largely attributed to the formation of covalent disulfide bonds between individual proteins and subsequent aggregation at neutral or acidic pH, due to its high free sulfhydryl content from sulfur-containing amino acids.

Tek V. and Zolkiewski M. (2002) Stability and interactions of the amino-terminal domain of ClpB from *Escherichia coli*. *Protein Sci* **11**, 1192-1198.

Abstract: ClpB is a member of a multichaperone system in *Escherichia coli* (with DnaK, DnaJ, and GrpE) that reactivates aggregated proteins. The sequence of ClpB contains two ATP-binding regions that are enclosed between the N- and C-terminal extensions. Whereas it has been found that the N-terminal region of ClpB is essential for the chaperone activity, the structure of this region is not known, and its biochemical properties have not been studied. We expressed and purified the N-terminal fragment of ClpB (residues 1-147). Circular dichroism of the isolated N-terminal region showed a high content of alpha-helical structure. Differential scanning calorimetry showed that the N-terminal region of ClpB is thermodynamically stable and contains a single folding domain. The N-terminal domain is monomeric, as determined by gel-filtration chromatography, and the elution profile of the N-terminal domain does not change in the presence of the N-terminally truncated ClpB (ClpB Δ N). This indicates that the N-terminal domain does not form strong contacts with ClpB Δ N. Consistently, addition of the separated N-terminal domain does not reverse an inhibition of ATPase activity of ClpB Δ N in the presence of casein. As shown by ELISA measurements, full-length ClpB and ClpB Δ N bind protein substrates (casein, inactivated luciferase) with similar affinity. We also found that the isolated N-terminal domain of ClpB interacts with heat-inactivated luciferase. Taken together, our results indicate that the N-terminal fragment of ClpB forms a distinct domain that is not strongly associated with the ClpB core and is not required for ClpB interactions with other proteins, but may be involved in recognition of protein substrates.

Top A., Kiick K. L. and Roberts C. J. (2008) Modulation of self-association and subsequent fibril formation in an alanine-rich helical polypeptide. *Biomacromolecules*. **9**, 1595-1603.

Abstract: Thermal unfolding, reversible self-association, and irreversible aggregation were investigated for an alanine-rich helical polypeptide, 17-H-6, with sequence [AAAQEAAAQAAAQAEAAQAAQ] 6. Dynamic light scattering, transmission electron microscopy, and thermal unfolding measurements indicate that 17-H-6 spontaneously and reversibly self-associates at acidic pH and low temperature. The resulting multimers have a compact, globular morphology with an average hydrodynamic radius approximately 10-20 nm and reversibly dissociate to monomers upon an increase to pH 7.4. Both free monomer and 17-H-6 chains within the multimers are alpha-helical and folded at low temperature. Reversible unfolding of the

monomer occurs upon heating of solutions at pH 7.4. At pH 2.3, heating first causes incomplete dissociation and unfolding of the constituent chains. Further incubation at elevated temperature induces additional structural and morphological changes and results in fibrils with a beta-sheet 2 degrees structure and a characteristic diameter of 5-10 nm (7 nm mean). The ability to modulate association and aggregation suggests opportunities for this class of polypeptides in nanotechnology and biomedical applications

Ulrih N. P., Anderluh G., Macek P., and Chalikian T. V. (2004) Salt-induced oligomerization of partially folded intermediates of equinatoxin II. *Biochemistry* **43**, 9536-9545.

Abstract: Equinatoxin II (EqTxII) is a cytolytic, water-soluble protein which binds to and forms cation-selective pores in lipid membranes. To characterize the native and denatured states of EqTxII and to elucidate the biological role of its oligomers, we have studied salt-dependent heat-induced conformational transitions of EqTxII. To this end, we have employed a variety of experimental techniques, including differential scanning calorimetry, circular dichroism and light absorption spectroscopy, ultrasonic velocimetry, electron microscopy, PAGE, and a hemolytic activity assay. This experimental combination has enabled us to monitor and structurally and thermodynamically characterize temperature-induced conformational transitions and oligomerization of EqTxII at different concentrations of NaCl. At pH 3.0 and 25 degrees C, EqTxII retains its native conformation and remains hemolytically active over a broad range of NaCl concentrations. However, an increase in the salt concentration results in a diminution of the thermal stability of EqTxII. Specifically, the calorimetrically determined denaturation temperature, T_d , and enthalpy, ΔH_{cal} , of the toxin decrease with an increase in the salt concentration. Our CD data suggest that the heat-induced denatured state of EqTxII lacks rigid tertiary structure while exhibiting well-defined secondary structure. The amount of the induced, non-native secondary structure of EqTxII depends on the solution ionic strength, temperature, time of incubation at an elevated temperature, and protein concentration. Our combined results suggest that, in the presence of salt, an increase in temperature results in formation of the partially unfolded state of the toxin that oligomerizes and forms biologically inactive, water-soluble aggregates.

Valery C., Artzner F., Robert B., Gulick T., Keller G., Grabielle-Madlmont C., Torres M. L., Cherif-Cheikh R., and Paternostre M. (2004) Self-association process of a peptide in solution: from beta-sheet filaments to large embedded nanotubes. *Biophys J* **86**, 2484-2501.

Abstract: Lanreotide is a synthetic octapeptide used in the therapy against acromegaly. When mixed with pure water at 10% (w/w), Lanreotide (acetate salt) forms liquid crystalline and monodisperse nanotubes with a radius of 120 Å. The molecular and supramolecular organization of these structures has been determined in a previous work as relying on the lateral association of 26 beta-sheet filaments made of peptide noncovalent dimers, the basic building blocks. The work presented here has been devoted to the corresponding self-association mechanisms, through the characterization of the Lanreotide structures formed in water, as a function of peptide (acetate salt) concentration (from 2% to 70% (w/w)) and temperature (from 15 degrees C to 70 degrees C). The corresponding states of water were also identified and quantified from the thermal behavior of water in the Lanreotide mixtures. At room temperature and below 3% (w/w) Lanreotide acetate in water, soluble aggregates were detected. From 3% to 20% (w/w) long individual and monodisperse nanotubes crystallized in a hexagonal lattice were evidenced. Their molecular and supramolecular organizations are identical to the ones characterized for the 10% (w/w) sample. Heating induces the dissolution of the nanotubes into soluble aggregates of the same structural characteristics as the room temperature ones. The solubilization temperature increases from 20 degrees C to 70 degrees C with the peptide concentration and reaches a plateau between 15% and 25% (w/w) in peptide. These aggregates are proposed to be the beta-sheet filaments that self-associate to build the walls of the nanotubes. Above 20% (w/w) of Lanreotide acetate in water, polydisperse embedded nanotubes are formed and the hexagonal lattice is lost. These embedded nanotubes exhibit the same molecular and supramolecular organizations as the individual monodisperse nanotubes formed at lower peptide concentration. The embedded nanotubes do not melt in the range of temperature studied indicating a higher thermodynamic stability than individual nanotubes. In parallel, the thermal behaviors of water in mixtures containing 2-80% (w/w) in peptide have been studied by differential scanning calorimetry, and three different types of water were characterized: 1), bulk water melting at 0 degrees C, 2), nonfreezing water, and 3), interfacial water melting below 0 degrees C. The domains of existence and coexistence of these different water states are related to the different Lanreotide supramolecular structures. All these results

were compiled into a binary Lanreotide-water phase diagram and allowed to propose a self-association mechanism of Lanreotide filaments into monodisperse individual nanotubes and embedded nanotubes.

van Teeffelen A. M., Meinders M. B., and de Jongh H. H. (2005) Identification of pitfalls in the analysis of heat capacity changes of beta-lactoglobulin A. *Int J Biol Macromol* **37**, 28-34.

Abstract: Information on changes in heat capacity (ΔC_p) of proteins upon unfolding is used frequently in literature to understand possible follow-up reactions of protein denaturation, like their aggregation propensity. This thermodynamic property is intrinsic to the protein's architecture and unfolding and should be independent of the approach used to evaluate it. However, for many proteins, the reported values for ΔC_p vary considerably. To identify whether the origin of these discrepancies lies within the experimental approach chosen and/or in the too simplified unfolding models used in the analysis of the data, we choose beta-lactoglobulin A, a relatively small protein, but disputed for its two-state unfolding, and established its ΔC_p from tryptophan fluorescence, near-UV circular dichroism and differential scanning calorimetric measurements. In view of the large variation for the obtained ΔC_p (between 3.2 and 10.1 \pm 0.8 kJ/(mol K)), it is evident that: (1) the sensitivity of different approaches to the structural changes; (2) irreversibility of unfolding; (3) non-ideal two-state unfolding behaviour need to be considered prior to interpretation. While the first two points can be addressed by using multiple approaches, the applicability of the selected unfolding behaviour for the analysis is often less easy to establish. In this work, we illustrate that by checking the wavelength-dependence used to detect protein conformational changes a tool is provided that gives a direct insight in the validity of the interpretation in these studies. An experimentally validated determination of ΔC_p allows a more proper use for the mechanistic understanding of protein denaturation and its follow-up reactions, avoiding pitfalls in the interpretation.

van Teeffelen A. M., Broersen K., and de Jongh H. H. (2005) Glucosylation of beta-lactoglobulin lowers the heat capacity change of unfolding; a unique way to affect protein thermodynamics. *Protein Sci* **14**, 2187-2194.

Abstract: Chemical glycosylation of proteins occurs in vivo spontaneously, especially under stress conditions, and has been linked in a number of cases to diseases related to protein denaturation and aggregation. It is the aim of this work to study the origin of the change in thermodynamic properties due to glucosylation of the folded beta-lactoglobulin A. Under mild conditions Maillard products can be formed by reaction of epsilon-amino groups of lysines with the reducing group of, in this case, glucose. The formed conjugates described here have an average degree of glycosylation of 82%. No impact of the glucosylation on the protein structure is detected, except that the Stokes radius was increased by approximately 3%. Although at ambient temperatures the change in Gibbs energy of unfolding is reduced by 20%, the denaturation temperature is increased by 5 degrees C. Using a combination of circular dichroism, fluorescence, and calorimetric approaches, it is shown that the change in heat capacity upon denaturation is reduced by 60% due to the glucosylation. Since in the denatured state the Stokes radius of the protein is not significantly smaller for the glucosylated protein, it is suggested that the nonpolar residues associate to the covalently linked sugar moiety in the unfolded state, thereby preventing their solvent exposure. In this way coupling of small reducing sugar moieties to solvent exposed groups of proteins offers an efficient and unique tool to deal with protein stability issues, relevant not only in nature but also for technological applications.

Vassall K. A., Stathopoulos P. B., Rumfeldt J. A., Lepock J. R., and Meiering E. M. (2006) Equilibrium thermodynamic analysis of amyotrophic lateral sclerosis-associated mutant apo Cu,Zn superoxide dismutases. *Biochemistry* **45**, 7366-7379.

Abstract: The folding and thermodynamic properties of metal free (apo) superoxide dismutases (SODs) are systematically analyzed using equilibrium guanidinium chloride (GdmCl) curves and differential scanning calorimetry (DSC). Chemically and structurally diverse amyotrophic lateral sclerosis (ALS)-associated mutations (G85R, G93R, E100G, I113T) are introduced into a pseudo-wild-type background that has no free cysteines, resulting in highly reversible unfolding. Analysis of the protein concentration dependence of GdmCl curves reveals formation of a monomer intermediate in equilibrium with native dimer and unfolded monomer. Global fitting of the data enables quantitative measurement of free energy changes for both dimer dissociation and monomer intermediate stability. All the mutations decrease protein stability, mainly by destabilizing the monomer intermediate, but also by tending to weaken dimerization, even for mutations far from the dimer interface. Thus, the effects of mutations seem to propagate through

the apo protein, and result in increased population of both intermediate and unfolded monomers. This may underlie increased formation of toxic aggregates by mutants in ALS. Analysis of DSC data for apo SODs is consistent with stability measurements from GdmCl curves and provides further evidence for increased aggregation by mutant proteins through increased ratios of van't Hoff to calorimetric enthalpies of unfolding.

Vega-Warner V. and Smith D. M. (2001) Denaturation and aggregation of myosin from two bovine muscle types. *J Agric Food Chem* **49**, 906-912.

Abstract: The thermal behaviors of myosin from bovine vastus intermedius (VI, predominantly red muscle) and semimembranosus (SM, predominantly white muscle) at pH 6.05 (ultimate pH of VI muscle) and 5.50 (ultimate pH of SM muscle) were compared. Differential scanning microcalorimetry and turbidity measurements were used to monitor changes in myosin during heating from 25 to 80 degrees C at 1 degrees C/min. VI and SM myosin heavy chain isoforms were identified on gradient SDS-PAGE. Endotherms of VI myosin at pH 6.05 had three transition temperatures (T_m) of 45, 53, and 57 degrees C, whereas at pH 5.50 two transitions were observed at 42 and 59 degrees C. SM myosin had two T_m values of 46 and 58 degrees C at pH 6.05 and T_m values of 43 and 62 degrees C at pH 5.5. SM myosin at its ultimate pH was less heat stable than VI myosin at its ultimate pH; however, when SM and VI myosin were compared at the same pH, VI myosin was less stable.

Verdino P., Keller W., Strohmaier H., Bischof K., Lindner H., and Koraimann G. (1999) The essential transfer protein TraM binds to DNA as a tetramer. *J Biol Chem* **274**, 37421-37428.

Abstract: The TraM proteins encoded by F-like plasmids are sequence specific DNA binding proteins that are essential for conjugative DNA transfer. We investigated the quaternary structure and the DNA binding properties of the TraM wild-type protein of the resistance plasmid R1 and two mutant forms thereof. Size-exclusion chromatography and differential scanning calorimetry showed that purified TraM protein (amino acids 2-127) forms stable tetramers in solution. A truncated version of the protein termed TraMM26 (amino acids 2-56) forms dimers. Thus, the dimerization and tetramerization domains can be assigned to the N-terminal and C-terminal domains of TraM, respectively. Further analyses using chemical cross-linking and light scattering corroborated the preferentially tetrameric nature of the protein but also suggest that TraM has a tendency to form higher aggregates. Band-shift and fluorescence spectroscopy investigations of TraM-DNA complexes revealed that the TraM protein is also tetrameric when bound to its minimal DNA binding site. The deduced binding constant in the range of 10^8 M^{-1} demonstrated a very strong binding of TraM to its preferred DNA sequence. Secondary structure analysis based on CD measurements showed that TraM is mainly alpha-helical with a significant increase in alpha-helicity (48 to 58%) upon DNA-binding, indicating an induced fit mechanism.

Verheul M., Roefs S. P., and de Kruif K. G. (1998) Aggregation of beta-lactoglobulin and influence of D2O. *FEBS Lett* **421**, 273-276.

Abstract: The conformational stability of beta-lactoglobulin increases in D2O over that in H2O. This is concluded from an increase in peak temperature by about 3 degrees C of differential scanning calorimetry (DSC) thermograms and from a decrease in overall aggregation rate. However, effects of pH and salt concentration on the heat-induced aggregation (reaction kinetics, DSC thermograms and aggregate growth) are similar in H2O and D2O. This indicates that the mechanism of heat-induced aggregation of beta-lactoglobulin is not significantly affected by replacement of H2O with D2O.

Vermeer A. W. and Norde W. (2000) The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. *Biophys J* **78**, 394-404.

Abstract: The denaturation of immunoglobulin G was studied by different calorimetric methods and circular dichroism spectroscopy. The thermogram of the immunoglobulin showed two main transitions that are a superimposition of distinct denaturation steps. It was shown that the two transitions have different sensitivities to changes in temperature and pH. The two peaks represent the F(ab) and F(c) fragments of the IgG molecule. The F(ab) fragment is most sensitive to heat treatment, whereas the F(c) fragment is most sensitive to decreasing pH. The transitions were independent, and the unfolding was immediately followed by an irreversible aggregation step. Below the unfolding temperature, the unfolding is the rate-determining step in the overall denaturation process. At higher temperatures where a relatively high concentration of (partially) unfolded IgG molecules is present, the rate of aggregation is so fast that IgG molecules become

locked in aggregates before they are completely denatured. Furthermore, the structure of the aggregates formed depends on the denaturation method. The circular dichroism spectrum of the IgG is also strongly affected by both heat treatment and low pH treatment. It was shown that a strong correlation exists between the denaturation transitions as observed by calorimetry and the changes in secondary structure derived from circular dichroism. After both heat- and low-pH-induced denaturation, a significant fraction of the secondary structure remains.

Villaverde J., Cladera J., Padros E., Rigaud J. L., and Dunach M. (1997) Effect of nucleotides on the thermal stability and on the deuteration kinetics of the thermophilic F₀F₁ ATP synthase. *Eur J Biochem* **244**, 441-448.

Abstract: Differential scanning calorimetry has been used to characterize the influence of specific nucleotide binding on the thermal unfolding of the F₀F₁-type ATP synthase from the thermophilic *Bacillus PS3* (TF₀F₁). The calorimetric trace shows an irreversible and kinetically controlled endothermic transition for TF₀F₁ in the absence of nucleotides. The thermal denaturation occurs at a transition temperature (*T_m*) of 81.7 degrees C. The remarkable thermostability of this enzyme was decreased upon tight binding of Mg²⁺ x ATP to noncatalytic sites, whereas binding of Mg²⁺ x ADP increased the temperature at which thermal denaturation occurred. At high temperatures, an exothermic transition due to aggregation processes was also affected by nucleotide binding. With the aim to correlate these thermal effects with possible structural differences among the various forms of TF₀F₁, Fourier transform infrared spectroscopy was carried out. Hydrogen/deuterium exchange was clearly affected by specific nucleotide occupancy. As illustrated by the total extent of protons exchanged, our results demonstrate that more peptide groups are exposed to the medium in the presence of Mg²⁺ x ATP than in the presence of Mg²⁺ x ADP. Therefore, consistent with microcalorimetric data, binding of Mg²⁺ x ADP induces conformational changes which shield amide protons to more buried hydrogen-bonded structures, whereas binding of Mg²⁺ x ATP results in a more open or flexible structure.

Visessanguan W., Ogawa M., Nakai S., and An H. (2000) Physicochemical changes and mechanism of heat-induced gelation of arrowtooth flounder myosin. *J Agric Food Chem* **48**, 1016-1023.

Abstract: Physicochemical changes of myosin during heating were investigated to elucidate the mechanism of heat-induced gelation of arrowtooth flounder (ATF) myosin at high ionic strength. Changes in dynamic properties indicated ATF myosin formed a gel in three different stages as shown by the first increase in G' (storage modulus) at 28 degrees C, followed by the decrease at 35 degrees C and the second increase at 42 degrees C. DSC thermogram showed the onset of myosin denaturation at 25 degrees C with two maximum transition temperatures at 30 and 36 degrees C. The decrease in alpha-helical content indicated ATF myosin began to unfold at 15 degrees C and the unfolding continued until it reached 65 degrees C. Turbidity measurement showed myosin began to aggregate at 23 degrees C and the aggregation was complete at 40 degrees C. Surface hydrophobicity increased consistently in the temperature range studied, 20-65 degrees C. Sulfhydryl contents decreased significantly at 20-30 degrees C due to the formation of disulfide linkages but remained constant at temperatures >30 degrees C. ATF myosin was shown to be extremely sensitive to heat, resulting in denaturation at lower temperature than other fish myosin. Denaturation was initiated by unfolding of the alpha-helical region in myosin followed by exposure of hydrophobic and sulfhydryl residues, which are subsequently involved in aggregation and gelation processes.

Vogl T. and Hinz H. J. (1994) Characterization of recombinant proteins: biochemical and biophysical criteria for the identity of native and renatured human tissue plasminogen activator and its mutants. *Biotechnol Appl Biochem* **20 (Pt 1)**, 1-22.

Abstract: Rapid progress in recombinant DNA techniques has led to the production of a great variety of native and modified proteins in bacteria which, however, frequently occur in non-native conformations or in aggregated form. Renaturation methods must necessarily include criteria for the confirmation of the native structure and function of the refolded macromolecules. This Review describes some of the spectroscopic and thermodynamic techniques which can provide powerful tests for the identity of native and renatured proteins. Emphasis was laid on the concentration requirements of the methods and on the ease of application and availability of instrumentation. As an example we used the single-chain recombinant tissue plasminogen activator (rt-PA) at different pH values and buffer systems. t-PA is a monomeric serine proteinase of M(r) 68,000 that converts plasminogen into plasmin, thereby promoting the

degradation of the fibrin network. This enzyme is a promising candidate for thrombolytic therapy of acute myocardial infarction. On the basis of the cDNA sequence the 527 amino acids of t-PA are likely to occur in five distinct structural domains. Specifically we exemplify the application of microcalorimetric studies for the verification of structural identity on rt-PA.

Waldner J. C., Lahr S. J., Edgell M. H., and Pielak G. J. (1999) Nonideality and protein thermal denaturation. *Biopolymers* **49**, 471-479.

Abstract: We studied the thermal denaturation of eglin c by using CD spectropolarimetry and differential scanning calorimetry (DSC). At low protein concentrations, denaturation is consistent with the classical two-state model. At concentrations greater than several hundred μM , however, the calorimetric enthalpy and the midpoint transition temperature increase with increasing protein concentration. These observations suggested the presence of intermediates and/or native state aggregation. However, the transitions are symmetric, suggesting that intermediates are absent, the DSC data do not fit models that include aggregation, and analytical ultracentrifugation (AUC) data show that native eglin c is monomeric. Instead, the AUC data show that eglin c solutions are nonideal. Analysis of the AUC data gives a second virial coefficient that is close to values calculated from theory and the DSC data are consistent with the behavior expected for nonideal solutions. We conclude that the concentration dependence is caused by differential nonideality of the native and denatured states. The nonideality arises from the high charge of the protein at acid pH and is exacerbated by low buffer concentrations. Our conclusion may explain differences between van't Hoff and calorimetric denaturation enthalpies observed for other proteins whose behavior is otherwise consistent with the classical two-state model.

Walasek P. and Honek J. F. (2005) Nonnatural amino acid incorporation into the methionine 214 position of the metzincin *Pseudomonas aeruginosa* alkaline protease. *BMC Biochem* **6**, 21.

Abstract: BACKGROUND: The alkaline protease from *Pseudomonas aeruginosa* (AprA) is a member of the metzincin superfamily of metalloendoproteases. A key feature of these proteases is a conserved methionine-containing 1,4-tight beta turn at the base of the active site zinc binding region. RESULTS: To explore the invariant methionine position in this class of protease, incorporation of a nonnatural fluorinated methionine, L-difluoromethionine (DFM), into this site was accomplished. Although overproduction of the N-terminal catalytic fragment of AprA resulted in protein aggregates which could not be resolved, successful heterologous production of the entire AprA was accomplished in the presence and absence of the nonnatural amino acid. DFM incorporation was found to only slightly alter the enzyme kinetics of AprA. In addition, differential scanning calorimetry indicated no significant alteration in the thermal stability of the modified enzyme. CONCLUSION: Although invariant in all metzincin proteases, the methionine 214 position in AprA can be successfully replaced by the nonnatural amino acid DFM resulting in little effect on protein structure and function. This study indicates that the increased size of the methyl group by the introduction of two fluorines is still sufficiently non-sterically demanding, and bodes well for the application of DFM to biophysical studies of protein structure and function in this class of protease.

Wang C., Eufemi M., Turano C., and Giartosio A. (1996) Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry* **35**, 7299-7307.

Abstract: To study the role of oligosaccharides on the properties of glycoproteins, five glycoproteins (yeast external invertase, bovine serum fetuin, glucoamylase from *Aspergillus niger*, and chicken egg white ovotransferrin and avidin) of previously established glycan patterns were purified to homogeneity and deglycosylated with endo- and exo-glycosidases in native conditions. Thermal stability and conformational changes were measured by high-resolution differential scanning microcalorimetry and circular dichroism spectroscopy before and after they were deglycosylated. It was found that deglycosylation decreases protein thermal stability, as judged by the decrease in denaturation temperature and denaturation enthalpy, while it does not affect substantially the conformation as indicated by the CD spectra in the far UV range. The destabilization effect of deglycosylation seems to depend on the carbohydrate content, i.e., the maximum effect was observed for the most heavily glycosylated protein, irrespective of the types (N-linked or O-linked) or patterns (mono- or multi-branched) of the covalently attached carbohydrate chains. In addition, studies of the reversibility to heat denaturation revealed that deglycosylated proteins have a poorer thermal reversibility in calorimetric scans than their native counterparts and tend to aggregate during thermal inactivation at acidic pH. These results suggest that carbohydrate moieties, in addition to the apparent stabilizing effect, may prevent the unfolded or partially folded protein molecules from

aggregation. Our results support the hypothesis that the general function of protein glycosylation is to aid in folding of the nascent polypeptide chain and in stabilization of the conformation of the mature glycoprotein.

Wang Q., Tolkach A., and Kulozik U. (2006) Quantitative assessment of thermal denaturation of bovine alpha-lactalbumin via low-intensity ultrasound, HPLC, and DSC. *J Agric Food Chem* **54**, 6501-6506.

Abstract: The degree of irreversible aggregation and the aggregation velocity constant of alpha-lactalbumin (alpha-la) were determined by three methods based on different principles: low-intensity ultrasound as a novel method for this purpose, DSC, and HPLC. The denaturation process of alpha-la causes a decrease in the ultrasonic velocity due to the conformation change of alpha-la molecules. This decrease is a function of the concentration of native alpha-la in the sample. A linear correlation was found between the degree of aggregation of alpha-la determined by these three methods. There is no significant difference between the aggregation velocity constants determined by the three methods. The results show that the ultrasonic method is capable of quantifying the degree of aggregation of a protein, offering an alternative method.

Waner M. J., Navrotskaya I., Bain A., Oldham E. D., and Mascotti D. P. (2004) Thermal and sodium dodecylsulfate induced transitions of streptavidin. *Biophys J* **87**, 2701-2713.

Abstract: The strong specific binding of streptavidin (SA) to biotin is utilized in numerous biotechnological applications. The SA tetramer is also known to exhibit significant stability, even in the presence of sodium dodecylsulfate (SDS). Despite its importance, relatively little is known about the nature of the thermal denaturation pathway for SA. This work uses a homogeneous SA preparation to expand on the data of previous literature reports, leading to the proposal of a model for temperature induced structural changes in SA. Temperature dependent data were obtained by SDS and native polyacrylamide gel electrophoresis (PAGE), differential scanning calorimetry (DSC), and fluorescence and ultraviolet (UV)-visible spectroscopy in the presence and absence of SDS. In addition to the development of this model, it is found that the major thermal transition of SA in 1% SDS is reversible. Finally, although SA exhibits significant precipitation at elevated temperatures in aqueous solution, inclusion of SDS acts to prevent SA aggregation.

Weijers M., Barneveld P. A., Cohen Stuart M. A., and Visschers R. W. (2003) Heat-induced denaturation and aggregation of ovalbumin at neutral pH described by irreversible first-order kinetics. *Protein Sci* **12**, 2693-2703.

Abstract: The heat-induced denaturation kinetics of two different sources of ovalbumin at pH 7 was studied by chromatography and differential scanning calorimetry. The kinetics was found to be independent of protein concentration and salt concentration, but was strongly dependent on temperature. For highly pure ovalbumin, the decrease in nondenatured native protein showed first-order dependence. The activation energy obtained with different techniques varied between 430 and 490 kJ*mole⁻¹. First-order behavior was studied in detail using differential scanning calorimetry. The calorimetric traces were irreversible and highly scan rate-dependent. The shape of the thermograms as well as the scan rate dependence can be explained by assuming that the thermal denaturation takes place according to a simplified kinetic process where N is the native state, D is denatured (or another final state) and k a first-order kinetic constant that changes with temperature, according to the Arrhenius equation. A kinetic model for the temperature-induced denaturation and aggregation of ovalbumin is presented. Commercially obtained ovalbumin was found to contain an intermediate-stable fraction (IS) of about 20% that was unable to form aggregates. The denaturation of this fraction did not satisfy first-order kinetics.

Weisel J. W. and Medved L. (2001) The structure and function of the alpha C domains of fibrinogen. *Ann N Y Acad Sci* **936**, 312-327.

Abstract: The alpha C domains have been localized on fibrinogen and fibrin. Several model systems have been developed to study their functions. Analysis of the amino acid sequence of the alpha C domains suggested that each is made up of a globular and an extended portion. Microcalorimetry confirmed this result and showed that the two alpha C domains interact intramolecularly. Electron microscopy of fibrinogen with a monoclonal antibody to the alpha C domains demonstrated that these regions normally interact with the central portion of the molecule. In the conversion from fibrinogen to fibrin there is a large scale conformational change, such that the alpha C domains dissociate from the central region and are

available for intermolecular interaction. Experiments with highly purified and well characterized fragment X monomer, missing either one or both of the alpha C domains, indicate that intermolecular interactions between alpha C domains are important for the enhancement of lateral aggregation during fibrin polymerization. Isolated alpha C fragments polymerized at neutral pH and interacted with the alpha C domains of fibrin monomer to influence clot formation. Several dysfibrinogenemias in which there are amino acid substitutions in, or truncations of, the alpha C domains revealed that these changes can have dramatic effects on polymerization and clot structure. The polymerization of A alpha 251 recombinant fibrinogen, that contains A alpha chains truncated at residue 251, was altered, as were the mechanical properties and the rate of fibrinolysis of the clots. Altogether, these results help to define the role of the alpha C domains in determining the structure and properties of clots.

Wenk M. and Jaenicke R. (1999) Calorimetric analysis of the Ca(2+)-binding betagamma-crystallin homolog protein S from *Myxococcus xanthus*: intrinsic stability and mutual stabilization of domains. *J Mol Biol* **293**, 117-124.

Abstract: The betagamma-crystallin superfamily consists of a class of homologous two-domain proteins with Greek-key fold. Protein S, a Ca(2+)-binding spore-coat protein from the soil bacterium *Myxococcus xanthus* exhibits a high degree of sequential and structural homology with gammaB-crystallin from the vertebrate eye lens. In contrast to gammaB-crystallin, which undergoes irreversible aggregation upon thermal unfolding, protein S folds reversibly and may therefore serve as a model in the investigation of the thermodynamic stability of the eye-lens crystallins. The thermal denaturation of recombinant protein S (PS) and its isolated domains was studied by differential scanning calorimetry in the absence and in the presence of Ca(2+) at varying pH. Ca(2+)-binding leads to a stabilization of PS and its domains and increases the cooperativity of their equilibrium unfolding transitions. The isolated N-terminal and C-terminal domains (NPS and CPS) obey the two-state model, independent of the pH and Ca(2+)-binding; in the case of PS, under all conditions, an equilibrium intermediate is populated. The first transition of PS may be assigned to the denaturation of the C-terminal domain and the loss of domain interactions, whereas the second one coincides with the denaturation of the isolated N-terminal domain. At pH 7.0, in the presence of Ca(2+), where PS exhibits maximal stability, the domain interactions at 20 degrees C contribute 20 kJ/mol to the overall stability of the intact protein.

Wicher K. B., Abou-Hachem M., Halldorsdottir S., Thorbjarnadottir S. H., Eggertsson G., Hreggvidsson G. O., Nordberg K. E., and Holst O. (2001) Deletion of a cytotoxic, N-terminal putative signal peptide results in a significant increase in production yields in *Escherichia coli* and improved specific activity of Cel12A from *Rhodothermus marinus*. *Appl Microbiol Biotechnol* **55**, 578-584.

Abstract: The thermostable cellulase Cel12A from *Rhodothermus marinus* was produced at extremely low levels when expressed in *Escherichia coli* and was cytotoxic to the cells. In addition, severe aggregation occurred when moderately high concentrations of the enzyme were heat-treated at 65 degrees C, the growth optimum of *R. marinus*. Sequence analysis revealed that the catalytic module of this enzyme is preceded by a typical linker sequence and a highly hydrophobic putative signal peptide. Two deletion mutants lacking this hydrophobic region were cloned and successfully expressed in *E. coli*. These results indicated that the N-terminal putative signal peptide was responsible for the toxicity of the full-length enzyme in the host organism. This was further corroborated by cloning and expressing the hydrophobic N-terminal domain in *E. coli*, which resulted in extensive cell lysis. The deletion mutants, made up of either the catalytic module of Cel12A or the catalytic module and the putative linker sequence, were characterised and their properties compared to those of the full-length enzyme. The specific activity of the mutants was approximately three-fold higher than that of the full-length enzyme. Both mutant proteins were highly thermostable, with half-lives exceeding 2 h at 90 degrees C and unfolding temperatures up to 103 degrees C.

Yamaoka T., Tamura T., Seto Y., Tada T., Kunugi S., and Tirrell D. A. (2003) Mechanism for the phase transition of a genetically engineered elastin model peptide (VPGIG)₄₀ in aqueous solution. *Biomacromolecules* **4**, 1680-1685.

Abstract: The concentration dependence of the pressure- and temperature-induced cloud point transition (Pc and Tc, respectively) of aqueous solutions of an elastin-like polypeptide with a repeating pentapeptide Val-Pro-Gly-Ile-Gly sequence (MGLDGSMG(VPGIG)₄₀VPLE) was investigated by using apparent light scattering, differential scanning calorimetry, and circular dichroism methods. In addition, the effects of salts and surfactants on these properties were investigated. The Pc and Tc of the present peptide in aqueous

solution were strongly concentration dependent. The calorimetric measurements showed that the enthalpy of transitions was 300-400 kJ/mol, i.e., 7-10 kJ/mol per VPGIG pentamer. The T_c of the (VPGIG)₄₀ solution was highly affected by the addition of inert salts or SDS. The effects of salts were consistent with those observed in the lyotropic series or Hoffmeister series. The CD spectrum at low peptide concentrations indicated that the present peptide forms type II beta-turn-like structure(s) at higher temperatures, but the temperature dependence of random coil diminishment (195 nm) and beta-turn formation (210 nm) were not exactly coincident. A hypothetical mechanism of the (VPGIG)₄₀ phase transition that could account for these observations was postulated. Observations suggest that the temperature-responsive properties of the elastin model peptides occur via a mechanism involving conformational change-association-aggregation and that the first two are strongly interactive.

Zhang Y., Roy S., Jones L. S., Krishnan S., Kerwin B. A., Chang B. S., Manning M. C., Randolph T. W., and Carpenter J. F. (2004) Mechanism for benzyl alcohol-induced aggregation of recombinant human interleukin-1 receptor antagonist in aqueous solution. *J Pharm Sci* **93**, 3076-3089.

Abstract: Benzyl alcohol, an antimicrobial preservative, accelerates aggregation and precipitation of recombinant human interleukin-1 receptor antagonist (rhIL-1ra) in aqueous solution. The loss of native monomer during incubation at 37 degrees C was determined by analysis of sample aliquots with size exclusion high performance liquid chromatography (SE-HPLC). Benzyl alcohol caused minor perturbation of the tertiary structure of the protein without changing its secondary structure, documenting that the preservative caused a minor shift in the protein molecular population toward partially unfolded species. Consistent with this conclusion, in the presence of benzyl alcohol the rate of H-D exchange was accelerated and the fluorescence of 1-anilinonaphthalene-8-sulfonic acid in the presence of rhIL1ra was increased. Benzyl alcohol did not alter the free energy of unfolding based on unfolding experiments in urea or guanidine HCl. With differential scanning calorimetry it was determined that benzyl alcohol reduced the apparent T_m of rhIL-1ra, but this effect occurred because the preservative lowered the temperature at which the protein aggregated during heating. Isothermal calorimetry documented that the interaction of benzyl alcohol with rhIL-1ra is relatively weak and hydrophobically driven. Thus, benzyl alcohol accelerates protein aggregation by binding to the protein and favoring an increase in the level of partially unfolded, aggregation-competent species. Sucrose partially inhibited benzyl alcohol-induced aggregation and tertiary structural change. Sucrose is preferentially excluded from the surface of the protein, favoring most compact native state species over expanded aggregation-prone forms.