

DSC XII– Amyloid and Prion Studies

Baxa U., Ross P. D., Wickner R. B., and Steven A. C. (2004) The N-terminal prion domain of Ure2p converts from an unfolded to a thermally resistant conformation upon filament formation. *J Mol Biol* **339**, 259-264.

Abstract: According to the "amyloid backbone" model of Ure2p prionogenesis, the N-terminal domain of Ure2p polymerizes to form an amyloid filament backbone surrounded by the C-terminal domains. The latter domains retain their native glutathione-S-transferase (GST)-like fold but are sterically inactivated from their regulatory role in nitrogen catabolism. We have tested this model by differential scanning calorimetry of soluble and filamentous Ure2p and of soluble C-terminal domains, combined with electron microscopy. As predicted, the C-terminal domains respond to thermal perturbation identically in all three states, exhibiting a single endotherm at 76 degrees C. In contrast, no thermal signal was associated with the N-terminal domains: in the soluble state of Ure2p, because they are unfolded; in the filamentous state, because their robust amyloid conformation resists heating to 100 degrees C.

Brender J. R., Hartman K., Reid K. R., Kennedy R. T. and Ramamoorthy A. (2008) A Single Mutation in the Nonamyloidogenic Region of Islet Amyloid Polypeptide Greatly Reduces Toxicity. *Biochemistry*. (publication)

Abstract: Islet amyloid polypeptide (IAPP or amylin) is a 37-residue peptide secreted with insulin by beta-cells in the islets of Langerhans. The aggregation of the peptide into either amyloid fibers or small soluble oligomers has been implicated in the death of beta-cells during type 2 diabetes through disruption of the cellular membrane. The actual form of the peptide responsible for beta-cell death has been a subject of controversy. Previous research has indicated that the N-terminal region of the peptide (residues 1-19) is primarily responsible for the membrane-disrupting effect of the hIAPP peptide and induces membrane disruption to a similar extent as the full-length peptide without forming amyloid fibers when bound to the membrane. The rat version of the peptide, which is both noncytotoxic and nonamyloidogenic, differs from the human peptide by only one amino acid residue: Arg18 in the rat version while His18 in the human version. To elucidate the effect of this difference, we have measured in this study the effects of the rat and human versions of IAPP 1-19 on islet cells and model membranes. Fluorescence microscopy shows a rapid increase in intracellular calcium levels of islet cells after the addition of hIAPP 1-19, indicating disruption of the cellular membrane, while the rat version of the IAPP 1-19 peptide is significantly less effective. Circular dichroism experiments and dye leakage assays on model liposomes show that rIAPP 1-19 is deficient in binding to and disrupting lipid membranes at low but not at high peptide to lipid ratios, indicating that the ability of rIAPP 1-19 to form small aggregates necessary for membrane binding and disruption is significantly less than hIAPP 1-19. At pH 6.0, where H18 is likely to be protonated, hIAPP 1-19 resembles rIAPP 1-19 in its ability to cause membrane disruption. Differential scanning calorimetry suggests a different mode of binding to the membrane for rIAPP 1-19 compared to hIAPP 1-19. Human IAPP 1-19 has a minimal effect on the phase transition of lipid vesicles, suggesting a membrane orientation of the peptide in which the mobility of the acyl chains of the membrane is relatively unaffected. Rat IAPP 1-19, however, has a strong effect on the phase transition of lipid vesicles at low concentrations, suggesting that the peptide does not easily insert into the membrane after binding to the surface. Our results indicate that the modulation of the peptide orientation in the membrane by His18 plays a key role in the toxicity of nonamyloidogenic forms of hIAPP

Casals C., Johansson H., Saenz A., Gustafsson M., Alfonso C., Nordling K. and Johansson J. (2008) C-terminal, endoplasmic reticulum-luminal domain of prosurfactant protein C - structural features and membrane interactions. *FEBS J* **275**, 536-547.

Abstract: Surfactant protein C (SP-C) constitutes the transmembrane part of prosurfactant protein C (proSP-C) and is alpha-helical in its native state. The C-terminal part of proSP-C (CTC) is localized in the endoplasmic reticulum lumen and binds to misfolded (beta-strand) SP-C, thereby preventing its aggregation and amyloid fibril formation. In this study, we investigated the structure of recombinant human CTC and the effects of CTC-membrane interaction on protein structure. CTC forms noncovalent trimers and supratrimeric oligomers. It contains two intrachain disulfide bridges, and its secondary structure is significantly affected by urea or heat only after disulfide reduction. The postulated Brichos domain of CTC, with homologs found in proteins associated with amyloid and proliferative disease, is up to 1000-fold more protected from limited proteolysis than the rest of CTC. The protein exposes hydrophobic surfaces, as

determined by CTC binding to the environment-sensitive fluorescent probe 1,1'-bis(4-anilino-5,5'-naphthalenesulfonate). Fluorescence energy transfer experiments further reveal close proximity between bound 1,1'-bis(4-anilino-5,5'-naphthalenesulfonate) and tyrosine residues in CTC, some of which are conserved in all Brichos domains. CTC binds to unilamellar phospholipid vesicles with low micromolar dissociation constants, and differential scanning calorimetry and CD analyses indicate that membrane-bound CTC is less structurally ordered than the unbound protein. The exposed hydrophobic surfaces and the structural disordering that result from interactions with phospholipid membranes suggest a mechanism whereby CTC binds to misfolded SP-C in the endoplasmic reticulum membrane

Chung C. M., Connors L. H., Benson M. D., and Walsh M. T. (2001) Biophysical analysis of normal transthyretin: implications for fibril formation in senile systemic amyloidosis. *Amyloid* **8**, 75-83.

Abstract: Transthyretin (TTR) is a plasma protein that transports thyroid hormone and retinol binding protein-vitamin A complex. Eighty-four variants of TTR have been identified and seventy-four are associated with familial amyloidotic polyneuropathy. Normal TTR is the major protein found in the fibrillar deposits in the heart at time of autopsy of individuals with senile systemic amyloidosis. The mechanism by which normally soluble TTR deposits as organ-damaging, insoluble, pathological fibrils late in life is unknown. Understanding the mechanism of fibrillogenesis of normal TTR is critical to the design of clinical treatments aimed at retardation, prevention, or reversal of fibril deposition. We have employed a biophysical approach to explore the hypothesis that an instability in a particular secondary or tertiary structure plays a role in the ability of normal TTR to form fibrils at physiological pH. Using far UV circular dichroic (CD) spectroscopy as a function of temperature we have identified simultaneous, cooperative, reversible structural changes in the beta-sheet and alpha-helical regions. The flexible short, surface-located loops undergo an irreversible conformational change at a lower temperature. Spectra before and after heating are different, particularly in the wavelength region associated with these loops, strongly suggesting that the major portion of TTR returns to its initial conformation while the loops do not. Near UV CD reveals partially reversible and irreversible changes in tertiary structure. Using calorimetry to directly measure the enthalpy associated with these changes, two peaks are observed, with further analysis suggesting conformational intermediates. Precipitates from heated samples reveal pre-fibrillar morphology by negative stain electron microscopy. These biophysical studies suggest that heat-induced conformational rearrangements enable normal TTR to assemble into pre-fibrils at physiological pH.

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

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

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

Abstract: A model cosolvent, ethanol, has profound and diversified effects on the amyloidogenic self-assembly of insulin, yielding spectroscopically and morphologically distinguishable forms of beta-aggregates. The alcohol reduces hydrodynamic radii of insulin molecules, decreases enthalpic costs associated with aggregation-prone intermediate states, and accelerates the aggregation itself. Increasing the concentration of the cosolvent promotes curved, amorphous, and finally donut-shaped forms. According to FT-IR data, inter-beta-strand hydrogen bonding is stronger in fibrils formed in the presence of ethanol. Mechanisms underlying the polymorphism of insulin aggregates were investigated by spectroscopic (CD, FT-IR, and fluorescence anisotropy) and calorimetric (DSC and PPC) methods. The nonmonotonic character of the influence of ethanol on insulin aggregation suggests that both preferential exclusion (predominant at the low concentrations) and direct alcohol-protein interactions are involved. The perturbed hydration of aggregation nuclei appears to be a decisive factor in selection of a dominant mode of beta-strand alignment. It may override unfavorable structural consequences of an alternative strand-to-strand stacking, such as strained hydrogen bonding. A hypothetical mechanism of inducing different amyloid "strains" has been put forward. The cooperative character of fibril assembly creates enormous energy barriers for any interstrain transition, which renders the energy landscape comblike-shaped.

Grage S. L., Afonin S., Grune M., and Ulrich A. S. (2004) Interaction of the fusogenic peptide B18 in its amyloid-state with lipid membranes studied by solid state NMR. *Chem Phys Lipids* **132**, 65-77.

Abstract: The interaction of the fusogenic polypeptide segment "B18" from the fertilization protein bindin with lipid membranes was investigated by solid state (2)H and (31)P NMR, and by differential scanning calorimetry. B18 is known to adopt different conformations depending on peptide concentration, ionic conditions, pH and lipid environment. Here, the peptide was studied in its beta-stranded amyloid conformation. According to (31)P NMR, the lamellar morphology of the DMPC bilayer remains intact in the presence of B18. In going from low (1:90) to high (1:10) peptide/lipid ratios, an increasing effect on several different (2)H-labeled lipid segments was observed, reflecting changes in phase behavior and local dynamics. The strongest influence of B18 was detected at the acyl-chains, while no significant effect on the lipid headgroup conformation was observed. This suggests an insertion of B18 in its fibrillar state into the membrane driven by hydrophobic interactions, rather than a peripheral binding mediated by electrostatics

Grasso D., Grasso G., Guantieri V., Impellizzeri G., La Rosa C., Milardi D., Micera G., Osz K., Pappalardo G., Rizzarelli E., Sanna D., and Sovago I. (2005) Environmental Effects on a Prion's Helix II Domain: Copper(II) and Membrane Interactions with PrP180-193 and Its Analogues. *Chemistry* **12**, 537-547.

Abstract: An abnormal interaction between copper and the prion protein is believed to play a pivotal role in the pathogenesis of prion diseases. Copper binding has been mainly attributed to the N-terminal domain of the prion protein, but this hypothesis has recently been challenged in some papers which suggest that the C-terminal domain might also compete for metal anchoring. In particular, the segment corresponding to the helix II region of the prion protein, namely PrP180-193, has been shown both to bind copper and to exhibit a copper-enhanced cytotoxicity, as well as to interact with artificial membranes. The present work is aimed at extending these results by choosing the most representative model of this domain and by determining its copper affinity. With this aim, the different role played by the electrostatic properties of the C- and N-termini of PrP180-193 (VNITIKQHTVTTTT) in determining its conformational behaviour, copper coordination and ability to perturb model membranes was investigated. Owing to the low solubility of PrP180-193, its copper affinity was evaluated by using the shorter PrPAc184-188NH(2) (IKQHT) analogue

as a model. ESI-MS, ESR, UV/Vis, and CD measurements were carried out on the copper(II)/PrPac184-188NH(2) and copper(II)/PrP180-193NH(2) systems, and showed that PrPac184-188NH(2) is a reliable model for the metal interaction with the helix II domain. The affinity of copper(II) for the helix II fragment is higher than that for the octarepeat and PrP106-126 peptides. Finally, the different ability of PrP180-193 analogues to perturb the DPPC model membrane was assessed by DSC measurements. The possible biological consequences of these findings are also discussed briefly.

Grasso D., Milardi D., La Rosa C., and Rizzarelli E. (2004) The different role of Cu⁺⁺ and Zn⁺⁺ ions in affecting the interaction of prion peptide PrP106-126 with model membranes. *Chem Commun (Camb)* 246-247.

Abstract: Differential scanning calorimetric (DSC) experiments have shown that the ability of PrP106-126 to perturb 1,3-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) model membranes is differently affected by Cu²⁺ and Zn²⁺ ions.

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.

Kazmirski S. L., Isaacson R. L., An C., Buckle A., Johnson C. M., Daggett V., and Fersht A. R. (2002) Loss of a metal-binding site in gelsolin leads to familial amyloidosis-Finnish type. *Nat Struct Biol* **9**, 112-116.

Abstract: Mutations in domain 2 (D2, residues 151-266) of the actin-binding protein gelsolin cause familial amyloidosis-Finnish type (FAF). These mutations, D187N or D187Y, lead to abnormal proteolysis of plasma gelsolin at residues 172-173 and a second hydrolysis at residue 243, resulting in an amyloidogenic fragment. Here we present the structure of human gelsolin D2 at 1.65 Å and find that Asp 187 is part of a Cd²⁺ metal-binding site. Two Ca²⁺ ions are required for a conformational transition of gelsolin to its active form. Differential scanning calorimetry (DSC) and molecular dynamics (MD) simulations suggest that the Cd²⁺-binding site in D2 is one of these two Ca²⁺-binding sites and is essential to the stability of D2. Mutation of Asp 187 to Asn disrupts Ca²⁺ binding in D2, leading to instabilities upon Ca²⁺ activation. These instabilities make the domain a target for aberrant proteolysis, thereby enacting the first step in the cascade leading to FAF.

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. $r = [\text{CR}]/[\text{protein dimer}]$, 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 $r = 0.3, 1.3, \text{ and } 4.8$, 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.

Litvinovich S. V., Brew S. A., Aota S., Akiyama S. K., Haudenschild C., and Ingham K. C. (1998) Formation of amyloid-like fibrils by self-association of a partially unfolded fibronectin type III module. *J Mol Biol* **280**, 245-258.

Abstract: The ninth type III module of murine fibronectin was expressed in *E. coli* and folded into a compact homogeneous monomer whose unfolding and refolding were then investigated by fluorescence, circular dichroism, calorimetry and electron microscopy. The isolated module is unusually labile under physiological conditions. When heated at 1 deg. C/minute it exhibits an irreversible endothermic transition between 35 and 42 degrees C depending on the protein concentration. The transition is accompanied by changes in secondary and tertiary structure with partial exposure of the single tryptophan and increased binding of the hydrophobic probe, 1,8-anilinonaphthalene-sulfonate. The partially unfolded intermediate undergoes rapid self-association leading to the formation of large stable multimers that, like the original monomer, contain substantial amounts of beta sheet structure. The multimers melt and dissociate reversibly in a second endothermic transition between 60 and 90 degrees C also depending on the protein concentration. This second transition destroys the remaining secondary structure and further exposes the tryptophan. Visualization of negatively stained specimens in the electron microscope reveals that partially unfolded rmIII-9 slowly forms amyloid-like fibrils of approximately 10 nm width and indeterminate length. A subdomain swapping mechanism is proposed in which beta strands from one partially unfolded molecule interact with complementary regions of another to form oligomers and polymers. The possibility that similar interactions could play a role in the formation of fibrils by fibronectin in vivo is discussed.

Mar Martinez-Senac M., Villalain J., and Gomez-Fernandez J. C. (1999) Structure of the Alzheimer beta-amyloid peptide (25-35) and its interaction with negatively charged phospholipid vesicles. *Eur J Biochem* **265**, 744-753.

Abstract: The secondary structure of amyloid betaAP(25-35) peptide was studied in pure form and in the presence of different phospholipid vesicles, by using Fourier transform infrared spectroscopy (FT-IR). Pure peptide aggregated with time, forming fibrils with beta-structure. Phospholipid vesicles formed by negatively charged phospholipids such as 1,2-dimyristoyl-sn-glycerol-3-phospho-L-serine (Myr2PtdSer), 1,2-dimyristoyl-sn-glycerol-3-phospho-rac-1-glycerol (Myr2PtdGro) and 1,2-dimyristoyl-sn-glycerol 3-phosphate (Myr2PtdH), greatly accelerated the aggregation of the peptide. However, the presence of vesicles formed by the zwitterionic phospholipid, 1, 2-dimyristoyl-sn-glycerol-3-phosphocholine (Myr2PtdCho), slowed down the aggregation process. Differential scanning calorimetry (DSC) measurements showed that the effect of betaAP(25-35) on the gel to crystal liquid phase transition was small at neutral pH for negatively charged phospholipids and practically nil for Myr2PtdCho. In the case of Myr2PtdSer the effect was also zero at pH 9 but the effect was large at pH 3. The effect on Myr2PtdH was not, however, very dependent on pH. These results were fully confirmed by the observation through FT-IR of the change with temperature of the CH₂ antisymmetric stretching vibration. The case of Myr2PtdGro was special as this phospholipid presents polymorphism giving solid quasicrystalline phases when it is not sufficiently hydrated, and it is remarkable that betaAP(25-35) was able to induce the formation of crystalline phases in samples prepared through a method which ensure a good hydration of phospholipid. These results show that the interaction of amyloid betaAP(25-35) peptide with phospholipids is based on electrostatic interactions, that these interactions favour the aggregation of the peptides, and that the presence of the aggregates may disturb the lipid-water interphase of the membrane.

Martsev S. P., Dubnovitsky A. P., Vlasov A. P., Hoshino M., Hasegawa K., Naiki H., and Goto Y. (2002) Amyloid fibril formation of the mouse V(L) domain at acidic pH. *Biochemistry* **41**, 3389-3395.

Abstract: The recombinant V(L) domain that represents the variable part of the light chain (type kappa) of mouse monoclonal antibody F11 directed against human spleen ferritin was found to form amyloid fibrils

at acidic pH as evidenced by electron microscopy, thioflavin T binding, and apple-green birefringence after Congo red staining. This is the first demonstration of amyloid fibril formation of the mouse V(L) domain. To understand the mechanism of acidic pH-induced amyloid fibril formation, conformational changes of the V(L) domain were studied by one-dimensional NMR, differential scanning calorimetry, analytical ultracentrifugation, hydrophobic dye binding, far-UV circular dichroism, and tryptophan fluorescence. The results indicated accumulation of two intermediate states during acid unfolding, which might be responsible for amyloid fibril formation. The more structured intermediate that exhibited maximal accumulation at pH 3 retained the natively-like secondary structure and a hydrophobic core, but exposed hydrophobic surfaces that bind 8-anilino-1-naphthalenesulfonate. Below pH 2, a more disordered intermediate with dequenched tryptophan fluorescence but still retaining the beta-sheet structure accumulated. The optimal pH of amyloid fibril formation (i.e., pH 4) was close to the optimal pH of the accumulation of the natively-like intermediate, suggesting that the amyloid fibrils might be formed through this intermediate.

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.

Olofsson A., Borowik T., Grobner G. and Sauer-Eriksson A. E. (2007) Negatively charged phospholipid membranes induce amyloid formation of medin via an alpha-helical intermediate. *J Mol Biol* **374**, 186-194.

Abstract: Medin, a recently discovered 5.5 kDa peptide, is associated with amyloid deposits in the medial layer of human arteries and the prevalence is nearly 100% within individuals above 50 years. Presently, not much is known about its biochemical and biophysical properties or its pathway from soluble peptide to insoluble amyloid. Here we have characterized the behavior of medin in the presence of lipid membranes, using circular dichroism, isothermal titration calorimetry, differential scanning calorimetry, size exclusion chromatography, and atomic force microscopy (AFM). Medin was shown to exist as a monomer in solution with a predominantly random-coil structure. It binds lipid vesicles that have either a neutral or a negative surface potential. Upon association to membranes containing acidic lipids, it undergoes an electrostatically driven conformational change towards a mainly alpha-helical state. Prolonged incubation converts medin from an alpha-helical structure into an amyloid beta-sheet fibrillar state as confirmed by AFM. Based on these findings, we propose a mechanism of medin-amyloid formation where medin electrostatically associates in its monomeric form to biological interfaces displaying a negative potential. This process both increases the local peptide concentration and induces an aggregation-prone alpha-helical fold.

Pappalardo G., Milardi D., Magri A., Attanasio F., Impellizzeri G., La R. C., Grasso D. and Rizzarelli E. (2007) Environmental Factors Differently Affect Human and Rat IAPP: Conformational Preferences and Membrane Interactions of IAPP17-29 Peptide Derivatives. *Chemistry* **13**, 10204-10215.

Abstract: Interest in the 37-residue human islet amyloid polypeptide (hIAPP) is related to its ability to form amyloid deposits in patients affected by type II diabetes. Attempts to unravel the molecular features of this disease have indicated several regions of this polypeptide to be responsible for either the ability to form insoluble fibrils or the abnormal interaction with membranes. To extend these studies to peptides that enclose His18, whose ionization state is believed to play a key role in the aggregation of hIAPP, we report on the synthesis of two peptides, hIAPP17-29 and rIAPP17-29, encompassing the 17-29 sequences of human and rat IAPP, respectively, as well as on their conformational features in water and in several membrane-mimicking environments as revealed by circular dichroism (CD) and 2D-NMR studies. hIAPP17-29 adopts a beta-sheet structure in water and its solubility increases at low pH. Anionic sodium dodecyl sulfate (SDS) micelles promoted the formation of an alpha-helical structure in the peptide chain, which was poorly influenced by pH variations. rIAPP17-29 was soluble and unstructured in all the environments investigated, with a negligible effect of pH. The membrane interactions of hIAPP17-29 and rIAPP17-29 were assessed by recording differential scanning calorimetry (DSC) measurements aimed at elucidating the peptide-induced changes in the thermotropic behaviour of zwitterionic (DPPC) and negatively charged (DPPC/DPPS 3:1) model membranes (DPPC=1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPS=1,2-dipalmitoyl-sn-glycero-3-phosphoserine). Results of DSC experiments demonstrated the high potential of hIAPP17-29 to interact with DPPC membranes. hIAPP17-29 exhibited a negligible affinity for negatively charged DPPC/DPPS model membranes at neutral pH. On the other hand, rIAPP17-29 did not interact with neutral or negatively charged membranes. The role played by His18 in the modulation of the biophysical properties of this hIAPP region was assessed by synthesising and studying the R18hIAPP17-29 peptide; the replacement of a single Arg with a His residue is not sufficient to induce either amyloidogenic propensity or membrane interaction in this region. The results show that the 17-29 domain of hIAPP has many properties of the full-length protein "in vitro" and this opens up new perspectives for both research and eventually therapy.

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.

Rezaei H., Choiset Y., Eghiaian F., Treguer E., Mentre P., Debey P., Grosclaude J., and Haertle T. (2002) Amyloidogenic unfolding intermediates differentiate sheep prion protein variants. *J Mol Biol* **322**, 799-814.

Abstract: Sheep is a unique example among mammalian species to present a strong correlation between genotype and prion disease susceptibility phenotype. Indeed a well-defined set of PrP polymorphisms at positions 136, 154 and 171 (sheep numbering) govern scrapie susceptibility, ranging from very high susceptibility for V136-R154-Q171 variant (VRQ) to resistance for A136-R154-R171 variant (ARR). To get better insight into the molecular mechanisms of scrapie susceptibility/resistance, the unfolding

pathways of the different full-length recombinant sheep prion protein variants were analysed by differential scanning calorimetry in a wide range of pH. In the pH range 4.5-6.0, thermal unfolding occurs through a reversible one-step process while at pH <4.5 and >6.0 unfolding intermediates are formed, which are stable in the temperature range 65-80 degrees C. While these general behaviours are shared by all variants, VRQ and ARQ (susceptibility variants) show higher thermal stability than AHQ and ARR (resistance variants) and the formation of their unfolding intermediates requires higher activation energy than in the case of AHQ and ARR. Furthermore, secondary structures of the unfolding intermediates differentiate variants: ARR unfolding intermediate exhibits random coil structure, contrasting with the beta-sheet structure of VRQ and ARQ unfolding intermediates. The rate of the unfolding intermediate formation allows us to classify genetic variants along increasing scrapie susceptibility at pH 4.0, VRQ and ARQ rates being the highest. Rather poor correlation is observed at pH 7.2. Upon cooling, these intermediates refold into stable species, which are rich in beta-type secondary structures and, as revealed by thioflavin T fluorescence and electron microscopy, share amyloid characteristics. These results highlight the prion protein plasticity genetically modulated in sheep, and might provide a molecular basis for sheep predisposition to scrapie taking into account both thermodynamic stability and transconformation rate of prion protein.

Sasahara K., Naiki H., and Goto Y. (2005) Kinetically controlled thermal response of beta2-microglobulin amyloid fibrils. *J Mol Biol* **352**, 700-711.

Abstract: Calorimetric measurements were carried out using a differential scanning calorimeter in the temperature range from 10 to 120 degrees C for characterizing the thermal response of beta2-microglobulin amyloid fibrils. The thermograms of amyloid fibril solution showed a remarkably large decrease in heat capacity that was essentially released upon the thermal unfolding of the fibrils, in which the magnitude of negative heat capacity change was not explicable in terms of the current accessible surface area model of protein structural thermodynamics. The heat capacity-temperature curve of amyloid fibrils prior to the fibril unfolding exhibited an unusual dependence on the fibril concentration and the heating rate. Particularly, the heat needed to induce the thermal response was found to be linearly dependent on the heating rate, indicating that its thermal response is under a kinetic control and precluding the interpretation in terms of equilibrium thermodynamics. Furthermore, amyloid fibrils of amyloid beta peptides also exhibited a heating rate-dependent exothermic process before the fibril unfolding, indicating that the kinetically controlled thermal response may be a common phenomenon to amyloid fibrils. We suggest that the heating rate-dependent negative change in heat capacity is coupled to the association of amyloid fibrils with characteristic hydration pattern.

Sasahara K., Naiki H., and Goto Y. (2006) Exothermic effects observed upon heating of beta2-microglobulin monomers in the presence of amyloid seeds. *Biochemistry* **45**, 8760-8769.

Abstract: To understand the initial stages in the formation of amyloid fibrils of beta(2)-microglobulin, a protein responsible for dialysis-related amyloidosis, the effects of heat on the acid-unfolded monomer at pH 2.5 were studied. In the presence of a low concentration of seed fibrils, differential scanning calorimetric thermograms of acid-unfolded beta(2)-microglobulin monomers showed a large decrease in heat capacity with a sigmoidal temperature-dependence, which was subsequently released at higher temperature. Measurements of circular dichroism, atomic force microscopy, ultracentrifugation, and repeated differential scanning calorimetry indicated that the exothermic sigmoidal transition is accompanied by the conversion of about 12% of the monomeric beta(2)-microglobulin molecules into amyloid fibrils, which subsequently dissociate into monomers at high temperature. Interestingly, amyloid fibrils, formed partly after the sigmoidal transition, exhibited a heating rate-dependent, kinetically controlled thermal response, indicating that 12% of the total protein is enough to exhibit the unique thermal response. On the other hand, the salt-induced protofibrils did not show such a calorimetric response, indicating that the kinetic thermal response is unique to the particular structure of fibrils. Taken together, although the calorimetric behavior of amyloid fibrils remains elusive, it may be interpreted in terms of the effects of heat associated with the formation, the association, and the unfolding of fibrils, in which the interactions between specific beta-sheet structures and water molecules play a crucial role and are sensitively reflected in the heat capacity change in protein solution.

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.

Sasahara K., Yagi H., Naiki H. and Goto Y. (2007) Heat-triggered conversion of protofibrils into mature amyloid fibrils of beta2-microglobulin. *Biochemistry* **46**, 3286-3293.

Abstract: Heat-triggered conversion of the salt-induced thin and flexible protofibrils into well-organized thick and straight mature amyloid fibrils was achieved with beta2-microglobulin, a protein responsible for dialysis-related amyloidosis. First, protofibrils that formed spontaneously at pH 2.5 in the presence of 0.5 M NaCl were aggregated by agitating the solution. Second, the aggregated protofibrils were heated in a cell of a differential scanning calorimeter (DSC). The DSC thermogram showed an exothermic transition with sigmoidal temperature dependence, resulting in a remarkably large decrease in the heat capacity of the solution. Third, on the basis of electron microscopy together with circular dichroism spectroscopy, seeding experiments, and a thioflavin T binding assay, the sigmoidal transition was found to represent the conversion of protofibrils into mature amyloid fibrils. Furthermore, DSC thermograms obtained at various heating rates revealed that the transition curve depends on the heating rate, implying that the effects of heat associated with the conversion to the mature fibrils are kinetically controlled, precluding an interpretation in terms of equilibrium thermodynamics. Taken together, these results highlight the importance of the change in heat capacity in addressing the biological significance of interactions between solvent water and amyloid fibrils and, moreover, in detecting the formation of amyloid fibrils

Sciacca M. F., Pappalardo M., Milardi D., Grasso D. M. and La R. C. (2008) Calcium-activated membrane interaction of the islet amyloid polypeptide: implications in the pathogenesis of type II diabetes mellitus. *Arch Biochem Biophys* **477**, 291-298.

Abstract: The role played by Ca(2+) ions in the interaction of the human islet amyloid polypeptide (hIAPP) with model membranes has been investigated by differential scanning calorimetry (DSC) and circular dichroism (CD) experiments. In particular, the interaction of hIAPP and its rat isoform (rIAPP) with zwitterionic dipalmitoyl-phosphatidylcholine (DPPC), negatively charged dipalmitoyl-phosphatidylserine (DPPS) vesicles and with a 3:1 mixtures of them, has been studied in the presence of Ca(2+) ions. The experiments have evidenced that amorphous, soluble hIAPP assemblies interact with the hydrophobic core of DPPC bilayers. Conversely, the presence of Ca(2+) ions is necessary to activate a preferential interaction of hIAPP with the hydrophobic core of DPPS membranes. These findings support the hypothesis that an impaired cellular homeostasis of Ca(2+) ions may promote the insertion of hIAPP into the hydrophobic core of carrier vesicles which is thought to contribute to an eventual intracellular accumulation of beta-sheet rich hIAPP aggregates

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.

Shih P., Holland D. R., and Kirsch J. F. (1995) Thermal stability determinants of chicken egg-white lysozyme core mutants: hydrophobicity, packing volume, and conserved buried water molecules. *Protein Sci* **4**, 2050-2062.

Abstract: A series of 24 mutants was made in the buried core of chicken lysozyme at positions 40, 55, and 91. The midpoint temperature of thermal denaturation transition (T_m) values of these core constructs range from 60.9 to 77.3 degrees C, extending an earlier, more limited investigation on thermostability. The T_m values of variants containing conservative replacements for the wild type (WT) (Thr 40-Ile 55-Ser 91) triplet are linearly correlated with hydrophobicity ($r = 0.81$) and, to a lesser degree, with combined side-chain volume ($r = 0.75$). The X-ray structures of the S91A (1.9 Å) and I55L/S91T/D101S (1.7 Å) mutants are presented. The former amino acid change is found in duck and mammalian lysozymes, and the latter contains the most thermostable core triplet. A network of four conserved, buried water molecules is associated with the core. It is postulated that these water molecules significantly influence the mutational tolerance at the individual triplet positions. The pH dependence of T_m for the S91D mutant was compared with that of WT enzyme. The pKa of S91D is 1.2 units higher in the native than in the denatured state, corresponding to $\Delta \Delta G_{298} = 1.7$ kcal/mol. This is a low value for charge burial and likely reflects the moderating influence of the buried water molecules or a conformational change. Thermal and chemical denaturation and far UV CD spectroscopy were used to characterize the in vitro properties of I55T. This variant, which buries a hydroxyl group, has similar properties to those of the human amyloidogenic variant I56T.

Shnyrov V. L., Villar E., Zhadan G. G., Sanchez-Ruiz J. M., Quintas A., Saraiva M. J., and Brito R. M. (2000) Comparative calorimetric study of non-amyloidogenic and amyloidogenic variants of the homotetrameric protein transthyretin. *Biophys Chem* **88**, 61-67.

Abstract: Familial amyloidotic polyneuropathy (FAP) is an autosomal dominant hereditary type of amyloidosis involving amino acid substitutions in transthyretin (TTR). V30M-TTR is the most frequent variant, and L55P-TTR is the variant associated with the most aggressive form of FAP. The thermal stability of the wild-type, V30M-TTR, L55P-TTR and a non-amyloidogenic variant, T119M-TTR, was studied by high-sensitivity differential scanning calorimetry (DSC). The thermal unfolding of TTR is a spontaneous reversible process involving a highly co-operative transition between folded tetramers and unfolded monomers. All variants of transthyretin are very stable to the thermal unfolding that occurs at very high temperatures, most probably because of their oligomeric structure. The data presented in this work indicated that for the homotetrameric form of the wild-type TTR and its variants, the order of stability is as follows: wild-type TTR approximately > T119M-TTR > L55P-TTR > V30M-TTR, which does not correlate with their known amyloidogenic potential.

Takeuchi M., Mizuguchi M., Kouno T., Shinohara Y., Aizawa T., Demura M., Mori Y., Shinoda H. and Kawano K. (2007) Destabilization of transthyretin by pathogenic mutations in the DE loop. *Proteins* **66**, 716-725.

Abstract: Transthyretin single-amino-acid variants are responsible for familial amyloidotic polyneuropathy, in which transthyretin variants accumulate extracellularly in the form of fibrillar aggregates. We studied the structural stabilities of four transthyretin variants (L58H, L58R, T59K, and E61K), in which a positively charged amino acid is introduced in a loop region between the D- and E-strands. In addition to being located in the DE-loop, L58 and T59 are involved in the core of the transthyretin monomer. The L58H, L58R, and T59K substitutions destabilized transthyretin more than the

E61K mutation did, indicating that transthyretin is substantially destabilized by the substitution of residues located in both the DE-loop and the monomer core. By utilizing hydrogen-deuterium exchange and nuclear magnetic resonance, we demonstrated that residues in the G-strand and the loop between the A- and B-strands were destabilized by these pathogenic mutations in the DE loop. At the quaternary structural level, the DE-loop mutations destabilized the dimer-dimer contact area, which may lead to transient dissociation into a dimer. Our results suggest that the destabilization of the dimer-dimer interface and the monomer core is important for the amyloidogenesis of transthyretin.

Yutani K., Takayama G., Goda S., Yamagata Y., Maki S., Namba K., Tsunasawa S., and Ogasahara K. (2000) The process of amyloid-like fibril formation by methionine aminopeptidase from a hyperthermophile, *Pyrococcus furiosus*. *Biochemistry* **39**, 2769-2777.

Abstract: Amyloid is associated with serious diseases including Alzheimer's disease and senile-systemic amyloidosis due to misfolded proteins. In the course of study of the denaturation process of methionine aminopeptidase (MAP) from the hyperthermophile *P. furiosus*, we found that MAP forms amyloid-like fibrils, and we then investigated the mechanism of amyloid fibril formation. The kinetic experiments on denaturation monitored by CD at 222 nm indicated that MAP in the presence of 3.37 M GuHCl at pH 3.31 changed to a conformation containing a considerable content of beta-sheet structure after the destruction of the alpha-helical structure. MAP in this beta-rich conformation was highly associated, and its stability was remarkably high: the midpoint of the GuHCl denaturation curve was 4.82 M at pH 3.0, and a thermal transition was not observed up to 125 degrees C by calorimetry. The amyloid-like fibril formation of MAP was confirmed by Congo red staining with a typical peak at 542 nm in the difference spectrum, showing a cross-beta X-ray diffraction pattern with a clear sharp reflection at 4.7 Å and a characteristic unbranched fibrillar appearance with a length of about 1000 Å and a diameter of about 70 Å in the electron micrographs. Present results indicate that the amyloid-like form of MAP appears just after the protein is almost completely denatured, and even highly stable proteins can also form amyloid-like conformation under conditions where the denatured state of the protein is abundantly populated.