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# Thermodynamic Properties Underlying the α-Helix-to-β-Sheet Transition, Aggregation, and Amyloidogenesis of Polylysine as Probed by Calorimetry, Densimetry, and Ultrasound Velocimetry

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In this work, we performed a detailed thermodynamic study of an aggregation-prone polypeptide, polylysine, to gain a deeper insight into the scenario of physicochemical events during its unfolding, aggregation, and amyloidogenesis. The precise and simultaneous determination of the partial molar volume, the heat capacity, and the coefficients of thermal expansion, as well as adiabatic and isothermal compressibility of the protein upon unfolding and aggregation, yields a thermodynamic picture of the aggregation process highlighting the importance of volume fluctuations during unfolding and amyloidogenesis of proteins.

Since the formation of orderly aggregated proteins, amyloids, was found to be associated with several neurodegenerative developments such as Alzheimer's, Creutzfeldt-Jakob's, and Parkinson's diseases, the problem of protein aggregation has begun to receive marked attention and is meanwhile known to be a rather common phenomenon.[1] Partial protein volume and its temperature and pressure derivatives as well as enthalpy and volume fluctuations are the thermodynamic parameters determining protein stability, unfolding, and aggregation behavior, and can be monitored by differential scanning (DSC) and pressure perturbation (PPC) calorimetry, densimetry, and sound velocimetry.[2,3] To the best of our knowledge, this is the first work attempting, through such a combination of methods, to put forward a detailed, comprehensive, and complete thermodynamic description of an aggregating peptide linking its thermodynamics to the generic behavior of proteins implicated in conformational diseases.

Recently, studies of the role of fluctuations of proteins and their coupling to the surrounding solvent have received increasing interest.[4] Protein fluctuations permit conformational motions, such as flips of side chains and backbone motions, thus causing transitions among the various substates of the protein. The term "slaving" has been coined[4] according to the fact that the volume, enthalpy, and electrical dipole moment fluctuations in the hydration shell and of the exposed amino acid (a. a.) residues are coupled to the surrounding thermal bath. The solvent is thus considered an active participant in protein dynamics and folding. The fluctuations are given by the relevant susceptibilities, the heat capacity $C_p$ at constant pressure, and the isothermal compressibility, $\beta_T$: $\langle(\Delta H)^2\rangle = k_B T^2 m C_p$, $\langle(\Delta V)^2\rangle = k_B T V \beta_T$. Here, $k_B$ is the Boltzmann constant and $V$ and $m$ are the volume and mass of the protein, respectively. The susceptibility of the solvent, water, is larger than that of proteins: $C_p$ for water is ∼4.2 J $K^{-1}$ $g^{-1}$ and typically 1.3 J $K^{-1}$ $g^{-1}$ for proteins; $\beta_T$ is $\sim 4.5 \cdot 10^{-10}$ $Pa^{-1}$ for water and about $(1-2)\cdot 10^{-10}$ $Pa^{-1}$ for proteins.[3] Combined enthalpy and volume fluctuations are related to the coefficient of thermal expansion, $\langle(\Delta H)(\Delta V)\rangle = k_B T^2 V \alpha$.[5] Volume fluctuations are a measure for the flexibility of a protein, which is related to its packing, void volume, and solvational properties, and, hence, are related to its functional properties. Upon destabilization, unfolding, aggregation, and fibrillation of proteins, volume fluctuations are expected to play a significant role as well[6] and are therefore also discussed in this study.

There are several reasons that polylysine is an excellent, probably even the simplest, model for protein aggregation studies. It undergoes an α-helix-to-β-sheet transition, the hallmark of protein aggregation, and forms amyloid-like fibrils.[7] Second, the sequenceless character of the polypeptide allows one to explore the recently voiced hypothesis of the aggregation as a common generic feature of proteins as polymers taking place whenever native protein tertiary contacts are overruled by "polymer-like" main-chain interactions.[1]

Poly-D-lysine (PDL) of 27 kD molecular mass was purchased from Sigma, U.S.A. Samples were prepared by dissolving the polypeptide in $H_2O$ at 2 wt % concentration. The pH of the sample was adjusted to 11.6 with diluted NaOH. Density

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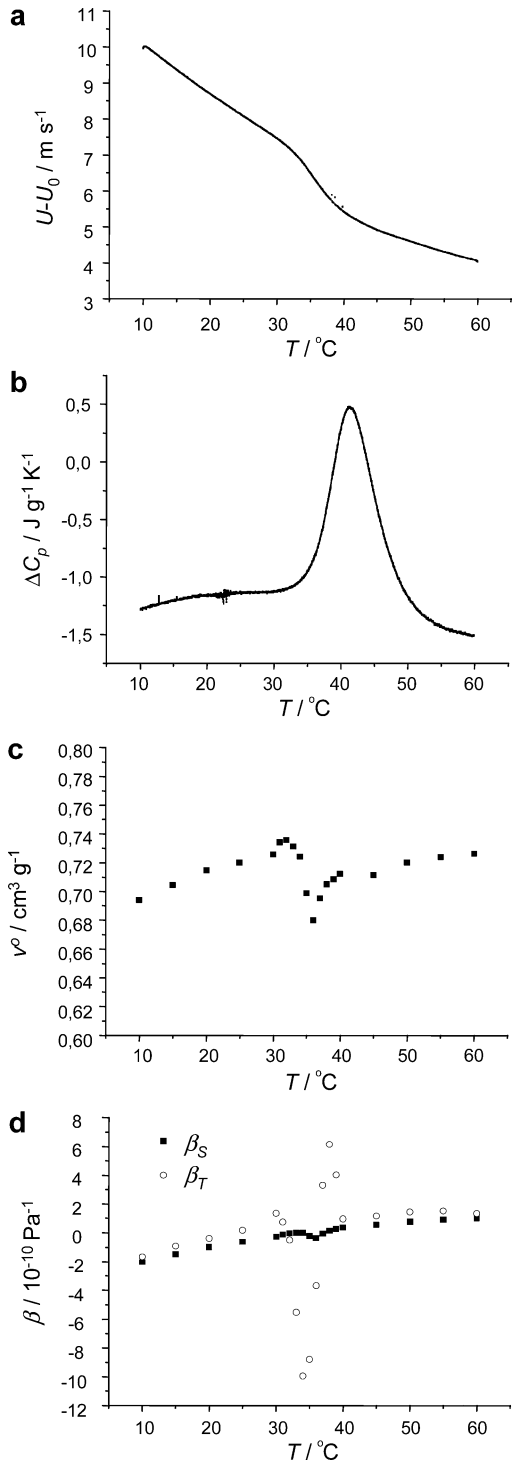


**Figure 1.** Results from measurements of the (a) ultrasound velocity, (b) heat capacity, (c) specific protein volume, and (d) adiabatic compressibility $\beta_S$ and isothermal compressibility $\beta_T$ of a 2 wt % polylysine solution at pH 11.6 as a function of temperature.

measurements were carried out with a DMA 58 vibrating tube densimeter with a precision of $\pm 5 \cdot 10^{-5}$ cm$^3$/g, which was calibrated for 20 different temperatures using air and water before every single experiment. Temperature was increased in a stepwise manner from 10 to 60 °C at a heating rate of 20 °C h$^{-1}$. The partial specific volume of the protein was calculated using $v^o = 1/c - (\rho - c)/\rho_0 c$ and normalized to 0.72 cm$^3$ g$^{-1}$ at 25 °C (Figure 1c). $\rho$ and $\rho_0$ are the densities of the solution and solvent, respectively; $c$ ($C$) is the specific (molar) concentration of the protein.

The most accurate method of determining the partial molar compressibility, $K_S^o$, of a solute is based on the Newton−Laplace equation, which relates the coefficient of adiabatic compressibility of a medium, $\beta_S = -(1/V)(\partial V/\partial p)_S$ with its density, $\rho$, and sound velocity, $U$: $U^2 = (\beta_S \rho)^{-1}$.[3] For (infinitely) dilute solutions, $K_S^o = \beta_{S0}(2V^o - 2[U] - M/\rho_0)$, where $K_S^o = V^o\beta_S$, $V^0 = (\partial V/\partial n)_{T,p}$ is the partial molar volume of the solute, $M$ its molar mass, and $[U] = (U - U_0)/(U_0 \cdot C)$ is the relative molar sound velocity (increment) of the solute; $U$ and $U_0$ are the sound velocities of the solute and solvent, respectively. The ultrasonic measurements were carried out using an ultrasonic resonator device (ResoScan system, TF Instruments GmbH, Heidelberg) with ultrasonic transducers made of single-crystal lithium niobate of a fundamental frequency of 9.5 MHz. The instrument comprises two independent cells for sample and reference with a path length of 7.0 mm. They are embedded into a metal block Peltier thermostat with a temperature stability of ±0.001 °C. The resolution of the ultrasonic velocity measurements is 0.001 m s$^{-1}$. Ultrasonic velocities of polylysine samples ($U$) and reference solvent ($U_0$) were measured over the same temperature range and at the same heating rate.

Experimental data on $\beta_T = K_T^o/V^o$ of proteins are very scarce, as it is technically difficult to measure the partial specific volume as a function of the pressure using densimetric techniques with high precision.[8] However, the partial molar isothermal compressibility of the solute can be obtained from the adiabatic value by $K_T^o = K_S^o + [T\alpha_0^2/(\rho_0 c_{p0})][2E^o/\alpha_0 - C_p^o/(\rho_0 c_{p0})]$, where $c_{p0}$ is the specific heat capacity at constant pressure of the solvent, $\alpha_0$ is the coefficient of thermal expansion of the solvent, respectively, and $E^o = (\partial V^o/\partial T)_p$ is the partial molar expansibility of the solute, which has been determined directly from the volumetric data shown is Figure 1c. The PPC data for $\alpha$ presented in the literature[7a] could not be used, because they have too few data points in the transition region and data averaging occurred on a different time scale. $C_p^o$ is the partial molar heat capacity of the solute, and $T$ is the absolute temperature. The heat capacity of the protein at constant pressure has been obtained from $C_p = \Delta C_p/m + v^o C_{p,0}/v_0^o$; $v^o$ and $v_0^o$ are the partial specific volumes of the solute and solvent, respectively.

Generally, small or negative values of $\beta_S$ were found for acid, base, and guanidinium hydrochloride induced unfolding reactions.[3] The experimental results have indicated that the compressibility of a protein can be divided into at least two components of opposite sign. Because of compressible cavities and voids in the protein interior, a first component is the protein intrinsic compressibility, $\beta_{S,intr}$, which is positive. The second term, $\beta_{S,hyd}$, is the contribution to the compressibility due to hydration and bound water. As the compressibility of single amino acids and small peptides in solution is negative,[3] $\beta_{S,hyd}$ is negative.

Calculations of the volume fluctuations of the system by using the expression $\langle(\Delta V)^2\rangle = k_B T V \beta_T$ implies that $V$ is the actual intrinsic volume of the protein, which represents the geometric volume of its solvent-inaccessible interior, and $\beta_T$ is the actual coefficient of isothermal compressibility of the protein interior.[3d] This equation cannot be directly used for partial molar volume or compressibility data as determined here, as these still contain large negative hydrational contributions. However, qualitative conclusions can be drawn.

Recently, we have shown using DSC (Figure 1b), PPC, and circular dichroism (CD) spectroscopic measurements[7a] that the $\alpha \rightarrow \beta$ transition in poly-D- and poly-L-lysine starts at ∼35 °C and is completed at 50 °C. As revealed by the increase of the

specific protein volume and the isothermal compressibility (Figures 1d), a temperature-induced destabilization of the polylysine's structure occurs just (~3 °C) before the onset of the unfolding and aggregation process. The increased isothermal compressibility data and hence mean-square volume fluctuations may result from an increased imperfect packing of the a. a. residues and the dynamic character of a partially destabilized polypeptide, which may be reminiscent of a molten globule kind of intermediate state, which has been shown to lead to a significant increase of compressibility values.[9] Upon unfolding and during the initial aggregation step, $\upsilon^o$, $\beta_S$, and most drastically $\beta_T$ decrease simultaneously, reaching minima around 35 °C. This can be ascribed to a release in void volume and—probably more importantly—an increased hydration of the protein surface, which is known to render $\beta_S$ negative. Generally, the higher the total solvent-accessible surface area, the more negative is the hydration contribution. Hence, the mean-square volume fluctuations increase slightly before the onset of the unfolding/aggregation reaction and then decrease drastically in a rather cooperative manner when the unfolding and aggregation process sets in. Upon subsequent aggregation and formation of mature fibrillar aggregates, as indicated by the CD and Fourier transform (FT)-IR spectroscopic data,[7a] significant dehydration occurs, whereby more densely packed water at the protein surface is released, leading to an increase of $\upsilon^o$, $\beta_S$, and $\beta_T$ again, which, at higher temperatures, are largely determined by the thermal volume effect.[9] The large increase of $\beta_T$ just after the transition (at ~38 °C) indicates that the early aggregates formed (which might well be still partially amorphous) still undergo large volume fluctuations, which rapidly decay upon formation of larger fibrils. The tight packing of $\beta$-sheets and depletion of internal cavities in the late fibrillar aggregates leads to a compaction and hence 20% reduction of the final value of the partial specific protein volume. The decrease in solvent-accessible area is also reflected in the negative $C_p$ change of $-4$ kJ mol$^{-1}$ K$^{-1}$ (Figure 1b). In comparison to $\beta_S$, $\beta_T$ is always slightly more positive above and below the transition and displays a drastically more pronounced temperature dependence in the transition region, however. The larger changes of $\beta_T$ may be attributed to the additional contribution of slower structural relaxation processes compared to $\beta_S$, which essentially reflects fast (hydrational) relaxation processes only. The slow structural relaxation processes detected by $\beta_T$ in the transition region may be due to structural relaxation processes of the highly flexible and dynamic transient oligomeric and smaller aggregate entities.

In conclusion, the precise and simultaneous determination of the thermodynamic variables $\upsilon^o$, $C_p$, $\alpha$, $\beta_S$, and $\beta_T$ upon unfolding and aggregation of polylysine, permits devising a unified thermodynamic picture of the aggregation process with highlighting the importance of volume fluctuations during unfolding and amyloidogenesis of proteins. The volume fluctuations appear to stem largely from changes in the flexibility of the protein, which is related to void volume and hydration changes during the various phases of the structural transformation.

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