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Differential Scanning Calorimetry Studies of the Inverse Temperature Transition of the Polypentapeptide of Elastin and Its Analogues

CHI-HAO LUAN, R. DEAN HARRIS, KARI U. PRASAD, and DAN W. URRY*

Laboratory of Molecular Biophysics, School of Medicine, University of Alabama at Birmingham, P.O. Box 300/University Station, Birmingham, Alabama 35294

SYNOPSIS

Differential scanning calorimetry studies have been carried out on the sequential polypeptide of elastin, (L-Val[1]-L-Pro[2]-Gly[3]-L-Val[4]-Gly[5])$_n$, abbreviated as PPP, and its more hydrophobic analogues (L-Leu[1]-L-Pro[2]-Gly[3]-L-Val[4]-Gly[5])$_n$, referred to as Leu[1]-PPP, and (L-Ile[1]-L-Pro[2]-Gly[3]-L-Val[4]-Gly[5])$_n$, referred to as Ile[1]-PPP. Consistent with inverse temperature transitions, the temperatures of the transitions for which maximum heat absorption occurs are inversely proportional to the hydrophobicities of the polypentapeptides (31°C for PPP, 16°C for Leu[1]-PPP, and 12°C for Ile[1]-PPP), and the endothermic heats of the transitions are small and increase with increasing hydrophobicity, i.e., *1.2, 2.9,* and *3.0* kcal/mol pentamer for PPP, Leu[1]-PPP, and Ile[1]-PPP, respectively. Previous physical characterizations of the polypentapeptides have demonstrated the occurrence of an inverse temperature transition since increase in order, as the temperature is raised above that of the transition, has been repeatedly observed using different physical characterizations. Furthermore, the studies demonstrated identical conformations for PPP and Ile[1]-PPP above and below the transition. Both heats and temperatures of the transitions vary with hydrophobicity, but not in simple proportionality.

INTRODUCTION

Elastin is the functional protein component of the biological elastic fiber. As the second most common protein in the extracellular matrix, it provides structurally vital elasticity to tissues such as lung, ligaments, vascular wall, and skin. Elastin is formed from a single precursor protein, tropoelastin, which becomes fibrous elastin on cross-linking of lysine side chains.[1-4] It has been found by Sandberg and by Rosenbloom and their colleagues[5,6] that tropoelastin contains repeating peptide sequences. The most striking repeating sequence is a polypentapeptide (PPP) that can be described as (L-Val[1]-L-Pro[2]-Gly[3]-L-Val[4]-Gly[5])$_n$, where n is 11 in the pig and cow, the latter occurring without a single substitution.[6] The sequential polypentapeptide has been synthesized and characterized by various physical and chemical methods in this laboratory.[7] When cross-linked (either chemically or by γ-irradiation), PPP forms an elastomeric matrix capable of an elastic modulus similar to that of fibrous elastin. A characteristic feature of the elastomeric PPP is that it undergoes an inverse temperature transition, i.e., the intramolecular and intermolecular order increases with an increase in temperature.[7] Since in the polypentapeptide (VPGVG)$_n$ there is a complete absence of polar side chains, the inverse temperature transition in this system provides a clearer perspective of the hydrophobic effect. Of further interest is that the synthetic PPP is soluble in water in all proportions below 25°C. When hydrophobic side chains of polypentapeptide are exposed to water, there is thought to be formation of a more-ordered interfacial (clathrate-like) water surrounding the hydrophobic side chains; after the inverse temperature transition, the more-ordered interfacial water

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* To whom correspondence should be addressed.

becomes less-ordered bulk water as hydrophobic side chains interact intra- and intermolecularly. The regular nonrandom association of hydrophobic side chains[8] results in an increase in order of the polypeptide part of the system. A molecular structure for the PPP, wherein intramolecular hydrophobic side-chain interactions are optimized as the PPP chain wraps up into a loose helical structure with interturn hydrophobic contacts,[8,9] has been put forward as the specific increase in intramolecular order of this polypeptide with increasing temperature.[7,10] The class of such helical structures has been called β-spirals.[11,12] The particular dynamic β-spiral of the PPP has repeating β-turns that function as spacers between turns of the helix with the interturn contacts utilizing dominantly the hydrophobic Val^1 and Pro^2 side chains.[13] The primary interturn hydrophobic contacts are between the Val^1 γCH_3 and Pro^2 βCH_2 moieties.[8] There is water within the β-spiral and suspended between β-turns is a peptide segment, Val^4–Gly^5–Val^1, which is largely surrounded by water. This segment can undergo large-amplitude low-frequency rocking motions, called librations.[14]

Another interesting analogue is Ile^1-PPP, (L-Ile^1–L-Pro^2–Gly^3–L-Val^4–Gly^5)$_n$. The only change from PPP is that instead of a valine in position 1 of the pentamer there is now an isoleucine residue. For each pentameric unit, the only change is a net increase of one CH_2 moiety. The third analogue studied is Leu^1-PPP, (L-Leu^1–L-Pro^2–Gly^3–L-Val^4–Gly^5)$_n$. The only change from PPP is that instead of a valine residue in position 1 of the pentamer, there is now a leucine residue. Again for each pentameric unit, the change is the addition of only one CH_2 moiety.

Differential scanning calorimetry (DSC) has been widely employed in the thermodynamic study of transitions that are initiated by either a decrease or an increase in temperature.[15] It provides a convenient method with which to characterize thermally induced transitions, and is used in the present study to determine and to compare the heats and temperatures of the inverse temperature transitions exhibited by PPP and its more hydrophobic analogues.

MATERIALS AND METHODS

The DSC studies were carried out on the DSC Micro 1710 (Hart Scientific, Provo, UT). The instrument includes four removable cells; one is used for reference and the other three can be used for sample. The thermopile response as a function of energy input was calibrated by the built-in calibration heater of the instrument. For each cell, a temperature-dependent calibration constant was determined every 10°C from −5 to 105°C and fit by the least-squares method to a second-order polynomial in temperature. And the polynomials are used in the experiment. The accuracy of the temperature measurement was calibrated in the factory, with a precision of ±0.5°C. Due to the instrument response time, the thermal profile depends on the scan rate used in the experiment. The transition temperature shift due to different scan rates can be estimated by the knowledge of the time constant, so that the zero scan-rate transition temperature can be obtained by extrapolation. As shown in Figure 1, diphenyl ether was used to check the performance of the instrument in temperature and heat of fusion measurements. In the calibration with diphenyl ether, a number of thermal scans have been run at different scan rates and the equation,

$$T_{\text{transition}}(\text{scan rate}) = \text{scan rate} \times \text{time constant} + T_{\text{transition}}(\text{zero scan rate})$$

was used to obtain the time constant of the instrument and the zero scan-rate transition temperature for the diphenyl ether by a least-squares fit. In 11 thermal scans of 8 different scan rates, the diphenyl ether, which has a melting point of 27.0°C and heat of fusion of 17.2 KJ/mol,[16] was observed to melt at 26.9 ± 0.1°C (with the melting temperature defined as the temperature at which the thermal profile departure from the pretransition baseline by an amount of 10% of the total transition) and to have a heat of fusion of 17.71 ± 0.05 KJ/mole. Conventionally, the temperature of maximum heat absorption T_m, is used as a characteristic parameter for a transition. From the observed T_m's for the diphenyl ether at different scan rates, the zero scan-rate T_m is obtained to be 27.8 ± 0.1°C. And the time constant of the instrument is approximately 4.6 min.

Although the transition temperature is scan-rate dependent, the transition enthalpy should not change with scan rate. This is demonstrated in the diphenyl ether runs and in the experiments with other compounds. The total heat of fusion for the 0.2 g diphenyl ether is at the level of 20 Joules, while the heats of transition of the polypentapeptides of elastin are about 1 Joule. Accordingly, another check of the instrument was carried out with ribonuclease A (Sigma, No. R-5000, from bovine pancreas type II-A, Activity 90) in glycine buffer with a total heat of transition less than 1 Joule. The sample preparation procedure of Schwarz and Kirchhoff[17] was

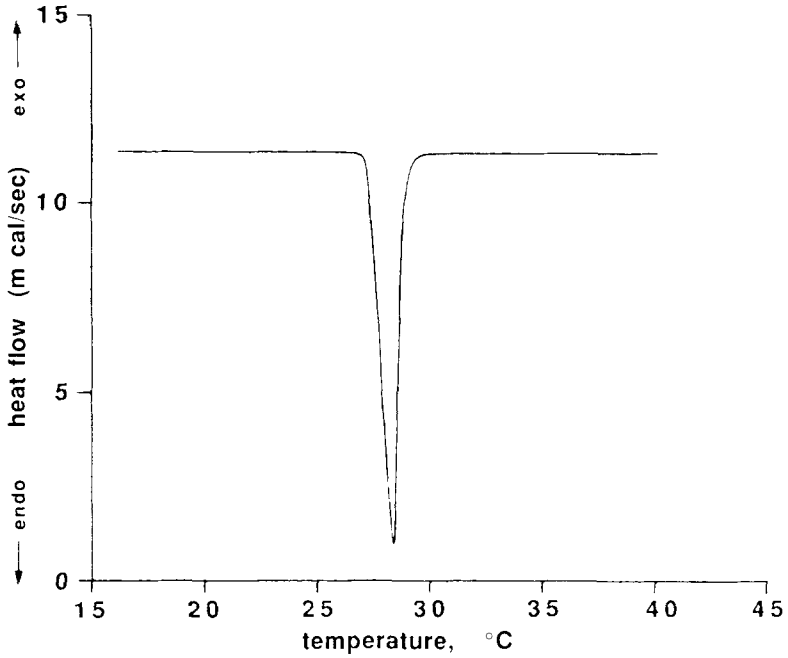

Figure 1 DSC scan of 0.226 g of diphenyl ether with a scan-rate of 7°C/h (.12°C/min). The transition half-width is 0.8°.

used. The results are shown in Figure 2. The zero scan-rate T_m's and ΔH's for ribonuclease A at two different pH values are (54.8°C, 3.81×10^2 KJ/mole for a solution at a 1.38 mass % concentration and at pH 3.1) and (60.4°C, 4.25×10^2 KJ/mol for 1.41 mass % concentration at pH 4.0), respectively. These values may be compared to previously reported values of (54.4°C, 4.03×10^2 KJ/mol for a 2.13 mass % concentration at pH 3.1) and (61.9°C, 4.46×10^2 KJ/mol for a 2.03 mass % concentration at pH 4.0).[17] The agreement is satisfactory, and the accuracy of the instrument is demonstrated both for the temperature and for the heats of transitions.

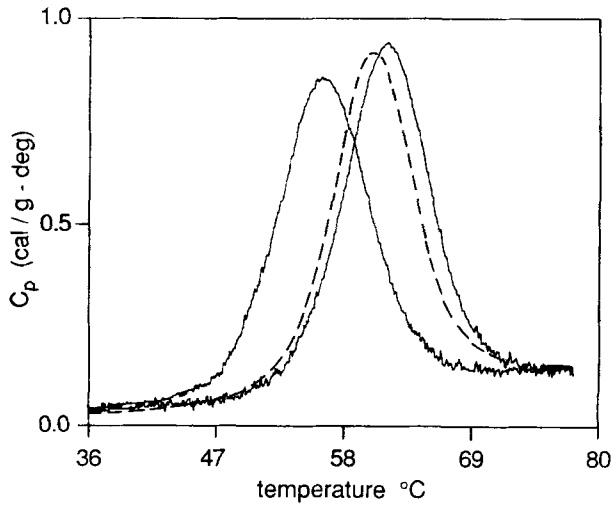

Figure 2 DSC scans of ribonuclease A in 0.2 *M* glycine buffer. The curve at left (solid line) is that for 1.38 mass % ribonuclease A in 0.2 *M* glycine buffer at pH 3.11 and the curve at right (solid line) is that for 1.41 mass % ribonuclease A in 0.2 *M* glycine buffer at pH 4.0. The middle curve (dashed line), replotted from Ref. 17 for the purpose of comparison, is that for 2.23 mass % ribonuclease A in 0.2 *M* glycine buffer at pH 3.87.

Preparation of the Polypentapeptides

The synthesis and characterization of PPP have been extensively reported elsewhere.[18,19] The syntheses and characterizations of Ile^1-PPP and of Leu^1-PPP have also been described previously.[20,21] The molecular weights of the three polypeptides are greater than 50,000 daltons.

Low-conductivity water (with a resistance greater than 15 MΩ) was used as the solvent for PPP, Ile^1-PPP, and Leu^1-PPP. This is referred to as type I water. In all the experiments, each weighed amount of sample was dissolved in the amount of water required to obtain a concentration of 40 mg polypeptide/mL of water, and set at low temperature (2°C) for about 12 h to ensure that the sample was completely dissolved in the solvent. The samples were preheated up to 70°C with a scan rate of 14°C/h (.23°C/min) such that the polypeptide systems will

have passed once through the inverse temperature transition before the data were recorded on the second run. The inverse temperature transition is found to be essentially reversible, but the reverse process is slow. The experiments began after the samples had reequilibrated at 2°C for ~ 90 h. The scan-rate used was again 14°C/h (0.23°C/min). A baseline scan with solvent follows each DSC scan involving sample. The sample scan and baseline scan were run at the same scan rate, and against the same reference cell, the content of which remained the same for the two runs. The reported curve is the result of subtracting the baseline run from the sample run.

RESULTS AND DISCUSSION

In Figure 3 are presented the thermal profiles for PPP, Ile[1]-PPP, and Leu[1]-PPP in water. Asymmetric transitions are observed for all three peptides. Such an asymmetric line shape could possibly be the result of a too rapid scan rate. This possibility was explored by using slower scan rates. The asymmetry of the thermal profile, however, was still observed in the experiments run at slower scan rates. This suggests that the asymmetry is an intrinsic property of the transition process under study. According to Sturtevant,[15] an asymmetric line shape observed in the DSC studies can be an indication that the transition is a process of molecular association. This, of course, is in accordance with the studies of the polypentapeptides of elastin by other methods, as mentioned in the introduction.

The DSC-derived thermodynamic parameters for the three polypentapeptides are listed in Table I. Both of the more hydrophobic analogues, Ile[1]-PPP and Leu[1]-PPP, exhibit lower characteristic transition temperatures (T_t) and both absorb more heat per gram (ΔQ) than does PPP.

In calculating ΔQ and ΔH, a major problem in any DSC study is establishing an appropriate reference (as contrasted with instrumental) baselines. The baseline used here is a straight line joining the initial and final temperatures of the transition, as schematically shown in Figure 3. The baselines are drawn from 3°C below the temperature at which the thermal profile departs abruptly from the pretransition baseline to a point tangent to the curve on the high-temperature side. The absolute value of ΔH, of course, depends on the baseline used, but this uncertainty in the value of the ΔH's of the three peptides is negligible when compared to the observed differences in magnitudes of ΔH for the different peptides.

The ΔQ, ΔH, ΔS, and T_t are calculated as follows:

$$\Delta Q = \int_{T_{initial}}^{T_{final}} \delta q(T) \qquad \Delta H = \Delta Q \times \mathrm{MW}$$

$$\Delta S = \int_{T_{initial}}^{T_{final}} \frac{\delta q(T)}{T} \quad \text{and} \quad T_t = \frac{\Delta H}{\Delta S}$$

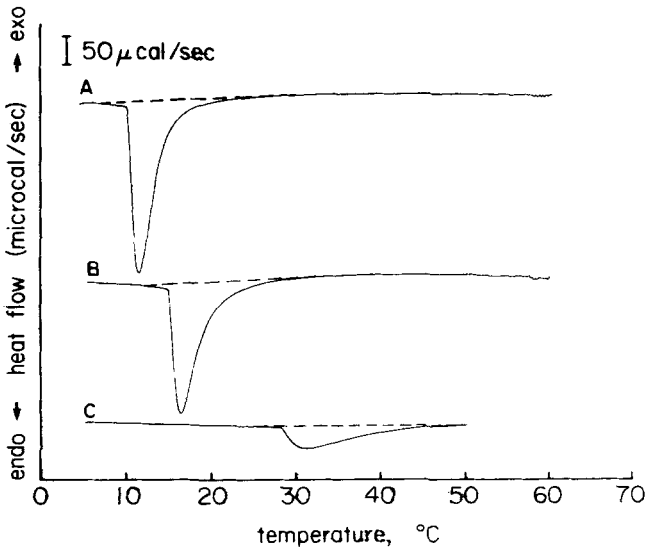

Figure 3 DSC scans for the 40 mg/mL solutions of the three synthetic polypeptides in H_2O: (A) Ile[1]-PPP, (B) Leu[1]-PPP, and (C) PPP. Each experiment was run at a scan rate of 0.23°C/min using 32 mg polypeptide.

Table I DSC-Derived Thermodynamic Data of the Inverse Temperature Transition Exhibited in Water by the Polypentapeptide and Its Analogues

Sample	T_m[a] (°C)	T_t (°C)	ΔH[b] (kcal/mol)	ΔS (cal/mol-K)	ΔQ (cal/g)
Ile[1]-PPP	11.6	12.9	3.0	10.4	7.02
Leu[1]-PPP	16.4	19.2	2.9	9.9	6.81
PPP	31.1	34.6	1.2	3.9	2.93

[a] T_m: temperature of maximum heat absorption. The reproducibility of the temperature is within ±0.1°C.
[b] The reproducibility of the heats are within ±0.1 kcal/mole.

Since the thermographs are obtained as heat absorption as a function of time, the ΔQ (ΔH) and ΔS are integrated with time as the integrand variable. In the DSC studies for each of the three polypeptide samples, endothermic processes are observed. Both the transition enthalpy and entropy are positive, which is indicative of an entropy-driven process.

Our immediate concern is the molecular process that gives rise to the heat absorption and the positive entropy change of the transition. This concern may be approached thermodynamically. Extensive thermodynamic studies of hydration of organic molecules in water have been reported, and the introduction of nonassociated nonpolar (hydrophobic) solutes into water is characterized by negative changes in enthalpy and entropy.[22-25] The negative enthalpy change means, of course, that the dissolution process is exothermic.

For the polypentapeptide–water system, at 20°C, which is below the transition temperature, the polypeptide is miscible with water in all proportions, and the exothermic dissolution in water has already taken place. Now on raising the temperature to 30°C, there occurs a reversible phase separation, i.e., a peptide-poor aqueous phase forms overlying a peptide-rich phase. The peptide-rich phase is called the coacervate, and the process is referred to as coacervation. The composition of the coacervate phase just above 30°C is about 40% peptide and 60% water by weight, i.e., about 400 mg/mL. When the concentration is just below 400 mg/mL, coacervation can be seen directly as the extrusion of water. Since the introduction of nonpolar groups into water is an enthalpy-driven (exothermic) process and consequently an entropy-decreasing process, the reverse process of removal of water from being in contact with nonpolar groups would be endothermic (i.e., an enthalpy- and entropy-increasing process). This allows that the observed endothermic process could indeed be one of the loss of solvation of apolar groups such as the loss of the interfacial (clathrate-like) water surrounding the hydrophobic side chains of the Val, Pro, Ile, and Leu residues. It is appreciated, however, that desolvation of polar groups could also be an endothermic process and result in a positive change in entropy. Fortunately for the present polypentapeptide system, extensive physical characterizations allow for substantive consideration of this issue, as will be discussed below.

The above discussion is a thermodynamic discussion with emphasis on the hydrophobic feature of the synthetic polypentapeptide of elastin with the associated interfacial (clathrate-like) water. A greater molecular understanding of the inverse temperature transition in the polypentapeptide–water system can be achieved by discussing in more detail the hydration of the polypentapeptide. The molecular mechanism of the inverse temperature transition of the polypentapeptide–water system has been suggested to rely on the special property of the waters of hydrophobic hydration in the system.[26,27] In this system, there are readily defined three different kinds of water. The first is the bulk water; the second involves the water molecules hydrogen bonding to the polar groups. Since there are no polar side chains in the three polypentapeptides, the backbone N-H and C=O moieties of the peptide are the only polar groups. In a Pro-containing pentamer unit there are four N-H and five C=O polar groups. There is a hydrogen bond between the residual one C=O and the Val[4] N-H both before and after the transition,[28] so that there are three N-H and four C=O groups left available to hydrogen bond with water or to other peptide moieties. The third kind of water is that interfacial water surrounding nonpolar groups. In PPP, Ile[1]-PPP, and Leu[1]-PPP, a striking characteristic is the preponderance of amino acids with nonpolar side chains. Water surrounding nonpolar groups is thought to form a cage of clathrate-like water with the above-noted interesting thermodynamic properties arising largely out of interwater hydrogen bonding.

The topic of clathrate-like water in relation to hydrophobic interactions has attracted much attention over the years.[29-32] With continuing studies, both experimental and theoretical, an understanding of the special structure and dynamic properties of water molecules surrounding nonpolar groups is under development. From the theoretical side, computer simulation studies on this issue have been carried out by a number of research groups (see, for example, Refs. 33–36). Simulations concerning solvation of nonpolar solutes in water and solvation of nonpolar groups in peptides demonstrate that there

is an increase in the structure of water molecules in the vicinity of a nonpolar solute. This means that these water molecules are in a lowered entropy state, and the interaction between these water molecules is stronger than that between water molecules in the absence of solute. This implies that the change from clathrate-like water to bulk water should be an endothermic process. The increased order of water surrounding nonpolar groups is reflected in the experimental finding that the reorientation correlation time for this water is slower than for bulk water[21,35] (see also C. Davis and D. W. Urry, unpublished microwave dielectric relaxation experiments). Most striking, of course, is the crystal structure of alkane gas hydrates where a pentagonal dodecahedron structure is observed for the interfacial water.[37] On the molecular level, therefore, it is the low-entropy and low-energy characteristics of the clathrate-like water, i.e., the water molecules surrounding the hydrophobic groups of the polypeptides, that determine the inverse temperature transition of the system. The driving force of the transition is derived, dominantly, from the low-entropy nature of the clathrate-like water, and the endothermic property of the transition is due to the conversion of the more strongly interacting clathrate-like water into bulk water.

In the above discussion, the transition of clathrate-like water to bulk water was considered; however, bulk water is not the only possible fate of the clathrate-like water after the transition. At a temperature above that of the transition, the polypeptides form β-spiral structures within which there is room for water to occur, and several β-spirals supercoil to form a high-order structure called a twisted filament, and there is also the possibility for water to occur between β-spirals and between twisted filaments.[7,10] This kind of water is, of course, not bulk water. Because the water molecules may be confined to intra- and intermolecular spaces, this water may be restricted in its behavior though in a manner that may be different from that of the clathrate-like water before the transition. It is appropriate to describe the endothermic inverse temperature transition process as the melting of more strongly interacting clathrate-like water into less restricted bulk water, and into nonbulk but less structured intraspiral and interspiral water.

In the above discussion, the contribution of the polypeptide itself to the free energy changes has been neglected. As demonstrated by many physical techniques,[7,10,27] for the polypeptide the transition is an ordering process, i.e., an entropy decreasing process. Also, low-frequency librational motions of the backbone peptide moieties develop within the peptide structure. This gives a positive entropy contribution to the transition, but since the librational frequency is localized and intense, it must occur as the result of the development of a regular structure.[38] Accordingly, the net entropy change of the polypeptide during the transition is not known. But strong evidence that the inverse temperature transition is mostly due to the solvent entropy change may be found in the comparison of the transitions of PPP, Leu^1-PPP, and Ile^1-PPP. Sequentially, the differences among the three polypeptides occur at position 1 of the pentamer unit; the residue at this position is valine, leucine, and isoleucine for PPP, Leu^1-PPP, and Ile^1-PPP, respectively. So the pentamers with isoleucine and leucine should be more hydrophobic than the pentamer with valine. Conformationally, the three polypeptides are essentially identical, i.e., containing a β-spiral with a type II Pro^2-Gly^3 β-turn utilizing a Val^1 (or Leu^1, or Ile^1) C=O $\cdots$ H-N Val^4 hydrogen bond.[39] It is reasonable to assume that the three polypeptides have very nearly the same entropy change when forming the β-spiral. The differences reside therefore in their relative hydrophobicities. So if the transitions are driven by hydrophobic interactions with the entropy difference mainly derived from the peptide nonpolar group–water interaction, the more hydrophobic polypeptides should show stronger hydrophobic interactions. The DSC data given in Figure 3 and Table I provide a clear affirmative answer to this prediction. The side chains of isoleucine and leucine have the same atomic composition, but isoleucine is generally considered more hydrophobic than leucine. This small difference is also demonstrated in the DSC studies of Ile^1-PPP and Leu^1-PPP. The change from valine to isoleucine or leucine is a net increase of one CH_2 moiety, but the change in the calorimetric result is dramatic. These differences are in the same order of their relative degree of hydrophobicity. In his experimental pioneering studies on hydrophobic hydration, Butler in 1937 arrived at the value for the ΔS of an added CH_2 moiety of 6 cal/mol-K.[40] Interestingly, as seen in Table I, this is essentially the differences in value obtained for the transition entropy on adding the CH_2 moiety as occurs when going from Val to Ile and to Leu, i.e., 6.5 cal/mol-K and 6.0 cal/mol-K, respectively.

What determines the transition temperature? Since $T_t = \Delta H/\Delta S$ holds in a true phase transition, T_t depends on the relative magnitudes of the increase in ΔH and of the increase in ΔS. The DSC studies show that both the ΔH's and the ΔS's increase with increasing hydrophobicity. This simply confirms

that larger, more hydrophobic groups have more clathrate-like water. But the studies also indicate that while the ΔH and ΔS increase with hydrophobicity of the polypeptide, the ratio of $\Delta H/\Delta S$, i.e. T_t, decreases with increasing hydrophobicity. This means that the change in ΔS for an increase in hydrophobicity is greater than the change in ΔH.

Accordingly, the transition temperature T_t reflects the ratio of $\Delta H/\Delta S$, with ΔH and ΔS dependent on the amount of clathrate-like water, which, of course, in turn, depends on the hydrophobicity of the polypeptide system. In the analogues presented, the change in ΔS is greater than the change in ΔH with increasing hydrophobicity of polypeptide.

As essentially all protein-directed life processes proceed with the involvement of water, the importance of hydrophobic interactions to biological processes appears to be fundamental, particularly when it comes to understanding details and driving forces of protein mechanisms. The folding of polypeptide chains into the native three-dimensional protein structure at physiological temperatures is generally a spontaneous process in a suitable solvent, usually a buffered aqueous medium. Hydrophobic interactions have been considered to be a major driving force in the protein folding and assembly process. Examples are the association of insulin molecules, the association of tobacco mosaic virus subunits, and the binding of chymotrypsin to substrate. But the natural protein has polar groups (charged and uncharged) and nonpolar (hydrophobic) groups. The forming and stabilizing of the native structure is the result of a balance of these various intra- and intermolecular interactions. In such a complex system it is hard, even impossible, to separate the effects of the hydrophobic interaction from the other interactions. In contrast, the synthetic polypentapeptide system provides a relatively simple but yet quite sophisticated system. Its simplicity makes it possible to separate the different contributions and to understand them on a molecular level, and yet its sophisticated nature allows examination of its behavior resulting from systematic changes both to the polypeptide and to the solvent, and to simulate what can happen in the biological system. It seems quite promising that the elucidation of the mechanism of the PPP system will contribute to an understanding of protein mechanisms in general. As a specific example, the polypentapeptide itself, and its appropriate modification, can exhibit mechanochemical coupling (chemomechanical transduction) in a predictable manner,[41,42] and in fact, is the first synthetic polypeptide system to exhibit such properties in the classical manner of doing the mechanical work of picking up and setting down weights as the result of a change in concentration of a chemical.

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