Energetics of the α-Lactalbumin States: A Calorimetric and Statistical Thermodynamic Study†

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ABSTRACT: The temperature dependence of the heat capacity function of holo and apo α-lactalbumin has been studied by high sensitivity differential scanning microcalorimetry. The heat capacities of the holo and apo forms in the native state were found to be close to, but somewhat higher than, that of lysozyme, which has a similar structure. At pH values higher than 5, the heat-denatured state and the unfolded state are indistinguishable. At lower pH values, the heat capacity of the state obtained by heat or acid denaturation is lower than what is expected for the completely unfolded polypeptide chain, but it approaches that value at higher temperatures. The heat capacity increment of the denatured state correlates well with the amount of residual structure measured by ellipticity (i.e., the lower the residual structure, the higher the heat capacity). The extent of residual structure in the denatured state, which is exceptionally high in α-lactalbumin, decreases upon increasing temperature and at ~110 °C becomes close to that observed in 6 M GdmCl. Above 110 °C, the denatured state of α-lactalbumin is practically indistinguishable in heat capacity and ellipticity from the fully unfolded state. The calorimetric data have been analyzed quantitatively using a statistically thermodynamic formalism. This analysis indicates that the long-range or global cooperativity of the protein is lost after heat denaturation of the native state, causing the remaining elements of residual structure to behave in a more or less independent fashion. At pH values close to neutral, heat denaturation occurs at high temperature and yields a totally unfolded polypeptide with no measurable population of partly folded intermediates. At lower pH values, denaturation occurs at lower temperatures and a progressively higher population of intermediates is observed. At pH 4.2, about 50% of the molecules is in compact intermediate states immediately after heat denaturation; however, at pH 3.5, this percentage is close to 80% and at pH 3.0 it reaches about 100% of the protein molecules. Upon heating, the unfolded state progressively becomes the predominant species. The analysis of the heat capacity data for α-lactalbumin indicates that the best model to account for the observed behavior is one in which the denatured state is represented as a distribution of substates with varying degrees of residual structure. At low temperatures, the distribution is centered around rather compact substates with significant residual structure. At higher temperatures, the distribution shifts toward states with less residual structure and eventually to the completely unfolded state.

The study of the conformational changes in α-lactalbumin (LA) led to the original formulation of the "molten globule" concept more than a decade ago (Kuwajima, 1977, 1989; Dolgikh et al., 1981, 1985; Ohigushi & Wada, 1983; Ptitsyn, 1992). According to this concept, a globular protein can exist not only in the compact native and the unfolded random coiled states but also in a state which is rather compact, has significant secondary structure, and has highly disrupted tertiary structure. This molten globule state gained attention because it is believed to represent a universal intermediate in protein folding (Ikeguchi et al., 1986; Kuwajima, 1989; Ptitsyn et al., 1990). Despite the existence of a large number of structural studies, the energetics of this state is still unclear because all attempts to measure calorimetrically the energies associated with the transition of the molten globule into the unfolded state have failed (Pfeil et al., 1986; Yutani & Kuwajima, 1992).

Indirect estimates of the thermodynamic parameters of GdmCl-induced unfolded of LA using the three-state model have led to controversial results. According to Kuwajima (1977) 63% of the unfolding enthalpy and entropy changes occur in the transition from the native to the intermediate (acidic) state and the remaining 37% between the intermediate and the unfolded state. Yutani et al. (1993) concluded that no enthalpy difference exists between the acidic intermediate and the unfolded state, i.e., that these two states are enthalpically equivalent. On the other hand, Xie et al. (1991) found that, for the GdmCl-induced intermediate at neutral pH, only 25% of the total enthalpy occurs between the native and the intermediate states at 25 °C.

In this paper, we show that the acid or heat-denatured state of α-lactalbumin (LA), which according to hydrodynamic, CD spectroscopy and heat capacity criteria retains significant residual structure, does unfold upon increasing temperature. The unfolding process of this molten globule structure is accompanied by a gradual increase in heat capacity. At approximately 110 °C, the heat capacity and ellipticity of the protein approach the limit expected for the fully unfolded polypeptide chain. Contrary to the heat denaturation of the native structure, which occurs as a highly cooperative two-state process, the unfolding of the heat-denatured state occurs over a wide temperature range in a rather noncooperative fashion. It is not clear, however, whether the observed heat capacity increase represents only the increased heat capacity of the unfolding polypeptide chain or also the additional diffuse heat absorption associated with the unfolding process.

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The denaturation of the native structure of globular proteins occurs with a change in the first derivative of the thermodynamic potential, i.e., with an enthalpy and an entropy change, and as such it can be considered as a first-order phase transition (albeit limited by its finite size) according to the classical Ehrenfest classification (Privalov, 1979, 1989). Higher-order phase transitions and in particular second-order phase transitions have been predicted for the unfolding of homopolypeptide chains whose compact state does not represent a unique structure (Lifshitz et al., 1978; Karplus & Shakhnovich, 1992). Proteins have unique structures defined by a hierarchical network of long- and short-range interactions that define high- and low-order cooperative elements (Wetlaufer, 1973; Rose, 1979; Freire & Murphy, 1991; Freire et al., 1992). High-order cooperative elements, which are responsible for the global cooperativity of the native protein, are destroyed during thermal denaturation. Under solvent conditions in which some low-order cooperative elements are more stable than higher-order ones, thermal denaturation does not lead to complete unfolding but to a state that exhibits varying degrees of residual structure. In this paper, we characterize the unfolding of these residual elements by analyzing the change in heat capacity that follows the heat denaturation transition. In the past, with less sensitive instrumentation, this gradual change in heat capacity could not be measured, and as such a thermodynamic analysis of the thermal unfolding of the heat-denatured state was not possible. The data presented in this paper have opened the doors for a rigorous analysis of the energetics of α-lactalbumin and the development of a statistical thermodynamic model for the folding/unfolding behavior of this and other proteins.

MATERIALS AND METHODS

Bovine α-lactalbumin (LA) was obtained from the milk of individual cows according to Armstrong et al. (1967). Purity of the preparation was controlled by electrophoresis in the native and denatured conditions (Laemmli, 1970; Ornstein, 1964). Apo-lactalbumin (aLA) was prepared by a demetallization procedure using a Chelex-100 column as described by Hiraoka and Sugai (1984). All reagents used were analytical grade.

The concentration of protein in solution was measured spectrophotometrically using an extinction coefficient $E_{1\%(280\ nm)}$ = 20.9 (Wetlaufer, 1961). The concentration of GdmCl in solution was determined from the refractive index at 589 nm (Pace, 1986).

CD measurements were carried out with a Jasco-710 spectropolarimeter equipped with thermostated cells.

Scanning calorimetric measurements of the heat capacity of LA in a broad temperature range were performed with a DASM-4 scanning microcalorimeter at a heating rate of 1 K/min. All measurements were carried out under an excess pressure of 3 atm, which permits expansion of the range of calorimetric measurements up to 120 °C. The protein concentration in these experiments ranged between 0.7 and 0.3 mg/mL. No concentration dependence of the partial heat capacity of the protein was observed in this concentration range. The partial specific heat capacity of the protein was determined as described elsewhere (Privalov & Potekhin, 1986) assuming that the molecular mass of LA is 14 260 kDa and partial specific volume is 0.709 cm^{-3} g^{-1}. The latter was calculated from the sequence of LA polypeptide chain using the known partial volumes of amino acid residues (Makhatadze & Privalov, 1990b).

The partial heat capacity of the polypeptide chain in the unfolded conformation was calculated according to Privalov and Makhatadze (1990), using the known heat capacity values of amino acid residues (Makhatadze & Privalov, 1990a) and assuming that in the extended conformation of the polypeptide chain all amino acid residues are exposed to water and contribute additively to the heat capacity.

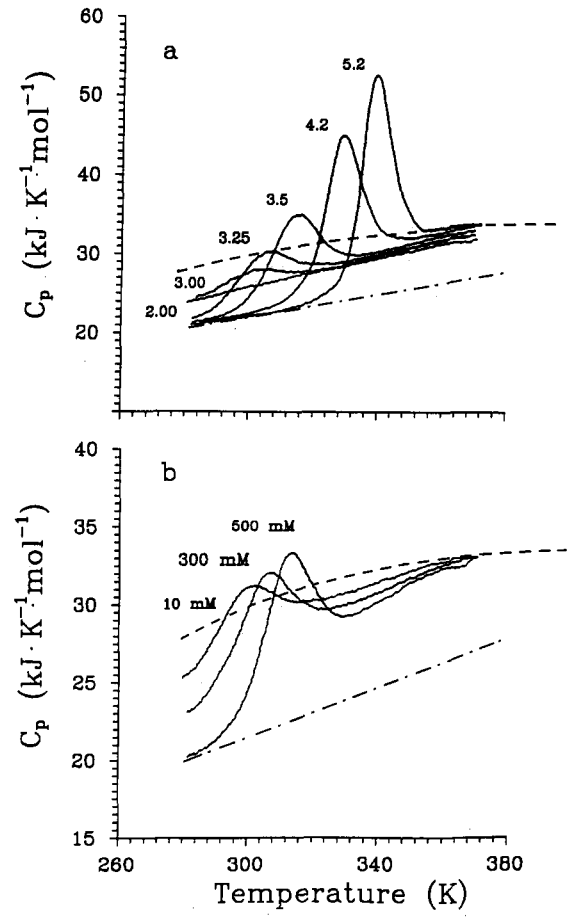

FIGURE 1: (Panel a) Temperature dependence of the partial heat capacity of LA in solutions with 10 mM NaAcO and Gly-HCl buffers at different pH values. (Panel b) Temperature dependence of the partial heat capacity of aLA in solutions with different concentration of Tris-HCl (molarity of buffer indicated on the curves). In both panels, the dashed line represents the heat capacity function calculated for the completely unfolded polypeptide chain of LA. The dot-and-dashed line represents the heat capacity function of the native state, assuming that it has the same slope as all other compact globular proteins, particularly native lysozyme.

Isothermal reaction calorimetric measurements were performed on the Jet-Titration microcalorimeter constructed at the Biocalorimetry Center of The Johns Hopkins University.

RESULTS

Heat Capacity. Figure 1 shows the temperature dependence of the partial heat capacity of holo-LA (panel a) as a function of pH and of aLA at pH 8 (panel b) as a function of the concentration of Tris-HCl. Additional experiments (not shown) indicate that, contrary to the apo form, the stability of the holo form is insensitive to variations in ionic strength at pH values close to neutrality. The stability of aLA is significantly less than that of holo-LA (Kuwajima et al., 1986) and decreases further upon decreasing the concentration of Tris-HCl (i.e., the ionic strength) or the pH of the solution as shown in Figure 2. At low pH values, the stability of aLA becomes very similar to that of LA. This is not surprising because at low pH values LA losses its ability to bind Ca^{2+} due to the protonation of three abnormally titrating carboxyl

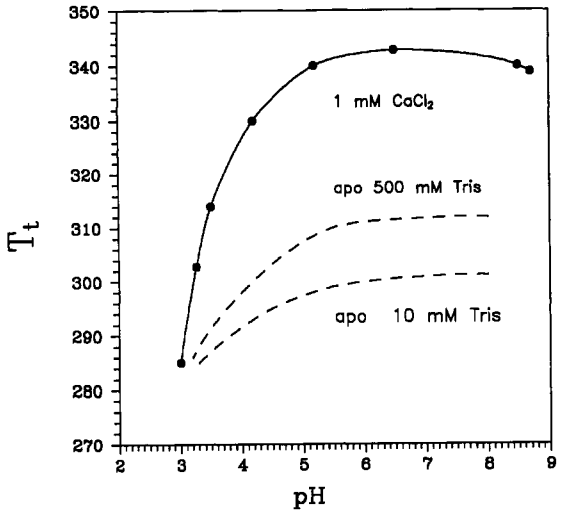

FIGURE 2: pH dependence of the denaturation temperature of LA in 1 mM $CaCl_2$ and aLA in solutions with 10 and 500 mM Tris-HCl.

groups responsible for the specific binding of Ca^{2+} (Kronman, 1989), i.e., at low pH values LA effectively converts into aLA.

The partial specific heat capacity of native LA is (1.40 ± 0.05) J K^{-1} g^{-1} at 20 °C, and its value does not appear to depend on pH when the protein is in the native state. The partial specific heat capacity of native LA is slightly higher than that of native lysozyme at a similar temperature, (1.30 ± 0.05) J K^{-1} g^{-1}. The difference between the heat capacities of LA and the structurally similar protein lysozyme may be a reflection of the somewhat looser structure of LA. According to crystallographic studies, the C terminus is flexible in human LA (Acharya et al., 1991) and the region between residues 104–111 appears to be highly fluctuating (Harata & Muraki, 1992). The heat capacity of native aLA is slightly higher than that of the holo form and equal to 1.55 ± 0.05 J K^{-1} g^{-1} at 20 °C. This might be considered as an indication that the apo form of the protein is more flexible than the holo form. However, the small difference in their heat capacity clearly shows that the removal of Ca^{2+} does not result in a significant expansion of the LA structure and a significant exposure of nonpolar groups to water.

As in the case of other globular proteins, the specific heat capacity of LA and aLA increases considerably upon thermal denaturation. In this case, however, the magnitude of the increase depends strongly on pH, temperature, and ionic conditions of the solution. At pH values greater than 5, the heat capacity increase is on the order of 7.48 kJ K^{-1} mol^{-1}. This value is very close to the value of 7.52 kJ K^{-1} mol^{-1}, expected for the complete unfolding of LA (Murphy et al., 1992; Haynie & Freire, 1993). Below pH 5, the magnitude of the increase in heat capacity is smaller than the value expected for complete unfolding but approaches that value with increasing temperature (see Figure 1). This is especially clear in the case of aLA because it denatures at a lower temperature where the difference between the heat capacities of the denatured and the completely unfolded states of LA is much more pronounced. Above 110 °C, the heat capacity of the acid and heat-denatured protein becomes very close to that of the unfolded polypeptide chain. Thus, the magnitude of the measured heat capacity changes indicate that the denaturation of α-lactalbumin is a complex process and that the nature of the denatured state depends on temperature, pH, and ionic conditions.

Enthalpy Change. The enthalpies for the heat denaturation of LA and aLA depend on the temperature at which

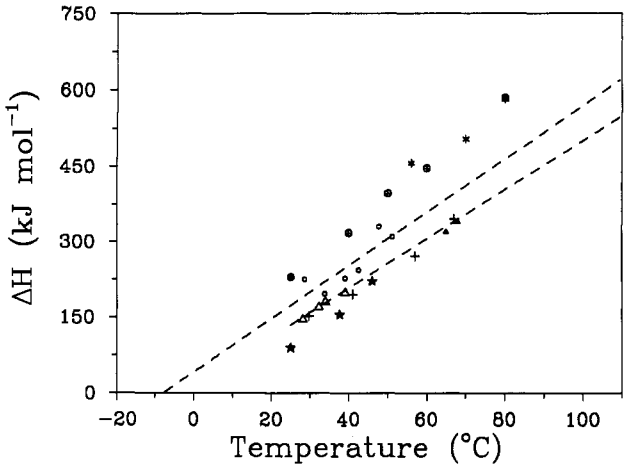

FIGURE 3: Temperature dependence of the enthalpy change associated with the thermal denaturation of LA and aLA in solutions with various concentration of Tris-HCl (Δ for apo form, ▲ for holo form) and acetate (+). (○) The enthalpies of aLA in 10 mM borate buffer pH 8.0 solutions with various concentration of NaCl measured by Pfeil and Sadowski (1985). (★) The enthalpy values obtained by acid titration of LA using isothermal reaction microcalorimetry. (⊕) The denaturation enthalpy of lysozyme obtained by reaction and (*) by scanning calorimetry (Pfeil & Privalov, 1976).

Table 1: Thermodynamic Characteristics of α-Lactalbumin and Apo-α-Lactalbumin Denaturation in Different Buffer Solutions

buffer (mM)	pH	T_t (K)	ΔH^{cal} (kJ/mol)	ΔH^{vh} (kJ/mol)	ΔC_p (kJ/K mol)	R^a
AcOH						
	5.2	340	340 ± 17	320 ± 16	7.46 ± 0.7	1.08
10	4.2	330	271 ± 14	250 ± 13	6.75 ± 0.7	1.01
	3.5	314	200 ± 10	215 ± 11	4.97 ± 0.5	0.94
	3.2	302.8	151 ± 15	182 ± 18	3.84 ± 0.4	0.84
Tris-HCl						
5	8.0	337.3	295 ± 15	289 ± 15	7.1 ± 0.7	1.02
500	8.0	338.8	315 ± 16	302 ± 15	7.9 ± 0.8	1.04
10 + 1 Ca	8.0	340.3	314 ± 16	304 ± 15	6.8 ± 0.7	1.03
apo-LA						
5	8.0	301.2	147 ± 26		6.18 ± 0.6	
300	8.0	305.7	172 ± 18	189 ± 19	5.34 ± 0.5	0.91
500	8.0	312.0	200 ± 10	195 ± 10	3.91 ± 0.4	1.09

[a] $R = \Delta H^{cal}/\Delta H^{vh}$.

denaturation occurs as judged by the area of the heat absorption peaks obtained under different solvent conditions (Figure 3 and Table 1). Two important features are apparent in Figure 3; first the enthalpy changes for LA and aLA are very similar indicating that the binding of Ca^{2+} is not accompanied by a significant enthalpic effect and, second, that the enthalpy change for LA is lower than the one observed for lysozyme. Also, the calorimetric and van't Hoff enthalpies for the main transition peaks are close to each other, indicating that the thermal denaturation of LA and aLA is a highly cooperative process. A similar temperature dependence of the heat effect of denaturation of LA is observed when denaturation is induced by acid and the heat effect is measured by isothermal titration microcalorimetry at various fixed temperatures (Figure 4). The fact that the enthalpy of heat and acid denaturation of LA is represented by the same temperature-dependent function confirms the earlier conclusion that the heat-denatured state of LA and its A state, which is obtained by decreasing pH and is usually called the molten globule state, are manifestations of the same macroscopic state of this protein (Pfeil & Sadowski, 1985; Dolgikh et al., 1985). This state is characterized by the absence of tertiary structure detectable by CD in the near-UV and by significant secondary structure content detectable by CD in the far-UV. It must be noted that the enthalpy

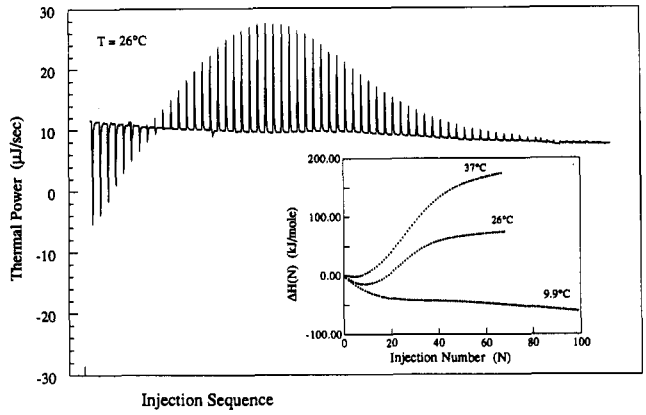

FIGURE 4: Heat effect observed during a microcalorimetric titration of LA at 26 °C and the enthalpy change, $\Delta H(N)$, of LA upon pH titration at 9.9, 26, and 37 °C as a function of N, the injection number. The enthalpy values obtained from these isothermal pH titrations coincide with the ones obtained by differential scanning calorimetry at equivalent temperatures.

change extrapolated to 110 °C is on the order of 580 ± 30 kJ mol^{-1}, i.e., ~43 J g^{-1}, instead of the value of 50 J g^{-1} obtained for other compact globular proteins and particularly lysozyme (Privalov, 1979). This phenomenon may also be a reflection of a looser structure in LA as discussed above.

CD Studies. The CD spectra in the near- and far-UV for aLA was measured under different solvent conditions and temperatures and are shown in Figure 5. The ellipticity at 270 nm (Figure 5a), which is considered as an index of the extent of tertiary structure in proteins, changes dramatically in the limited temperature region in which the denaturation heat absorption peak is observed calorimetrically. Upon heating solutions with different Tris-HCl concentrations, the ellipticity at 270 nm falls to a level which becomes independent of both Tris-HCl concentration and temperature. The ellipticity in the far-UV, which is considered to be an index of the secondary structure content in proteins, shows that aLA does not lose significant secondary structure upon denaturation; however, the residual secondary structure content in the denatured protein decreases gradually upon increasing the temperature [see also Kuwajima et al. (1985)]. As shown in Figure 5b, the ellipticity decreases upon increasing the temperature and at 110 °C, its value becomes close to that of LA in 6 M GdmCl. The value of the ellipticity at 222 nm and 110 °C, 5.0 × 10^3 deg cm^2 dmol^{-1} normalized on a per residue basis, appears to be universal for all proteins in the presence and absence of GdmCl (Privalov et al., 1989). Since it is hard to assume that the polypeptide chain retains any helical conformation at that temperature in 6 M GdmCl, this value of ellipticity at 222 nm might be considered, perhaps as corresponding to the random coiled conformation of the polypeptide chain. Thus, we arrive at the conclusion that the residual structure in LA, which is rather significant at room temperature, practically disappears upon heating to 110 °C. This change in ellipticity parallels the increase in heat capacity of the denatured state.

STATISTICAL THERMODYNAMIC FORMULATION OF THERMAL DENATURATION TRANSITION

Hierarchical Cooperative Model. The results presented above indicate that under certain conditions α-lactalbumin exhibits a two-stage thermal denaturation: a sharp cooperative denaturation process followed by a gradual unfolding of the protein. Under those conditions, the state existing immediately after thermal denaturation is not a fully unstructured and

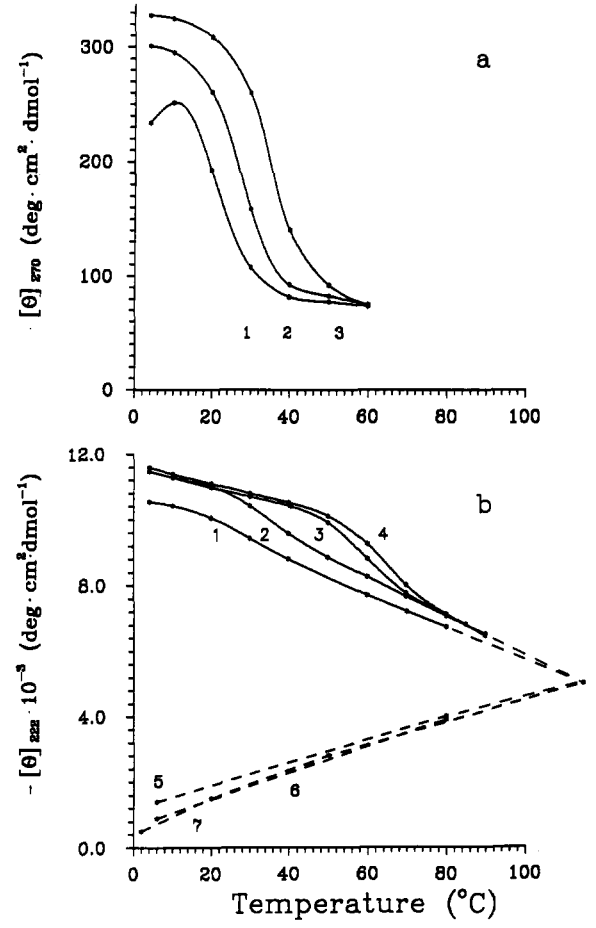

FIGURE 5: (Panel a) Temperature dependence of the ellipticity at 270 nm for aLA in solutions with various concentration of Tris-HCl at pH 8.0: (1) 10 mM, (2) 300 mM, and (3) 500 mM. (Panel b) Temperature dependence of the ellipticity at 222 nm for aLA in solutions with concentration of Tris-HCl 10 mM (1) and 500 mM (2) and for LA in solutions with 0.1 mM Ca^{2+} (3) and 1 mM Ca^{2+} (4). The dashed lines show the temperature dependence of the ellipticity in solutions containing 6 M GdmCl for lysozyme (-S-S) (5), apocytochrome c (6), and aLA (7).

hydrated random coil but rather a compact state exhibiting different degrees of secondary structure and a significant hydrophobic core as demonstrated by the heat capacity and ellipticity values. The degree of residual structure in this compact denatured or molten globule state is a function of temperature as well as solvent conditions (e.g., pH, ionic strength). Upon increasing the temperature, the compact denatured state progressively transforms into the unfolded state. When studied by differential scanning calorimetry, the transition from the folded state to the compact denatured state is seen as a highly cooperative process which approaches the behavior of a two-state transition, while the transition from the compact denatured state to the unfolded state occurs gradually and with a barely noticeable heat effect.

It can be assumed that the two-stage character of the thermal unfolding transition is a consequence of the existence of long- and short-range cooperative interactions within the protein molecule. The interactions within the native state of a protein can be used to define an intricate hierarchical network responsible for the long-range or global cooperative behavior of the molecule. At the most fundamental level of this hierarchical network there is an arbitrary number of structural elements with the ability to exhibit local cooperativity and folding behavior. Higher-order cooperative elements are formed by interactions between lower-order elements (Freire

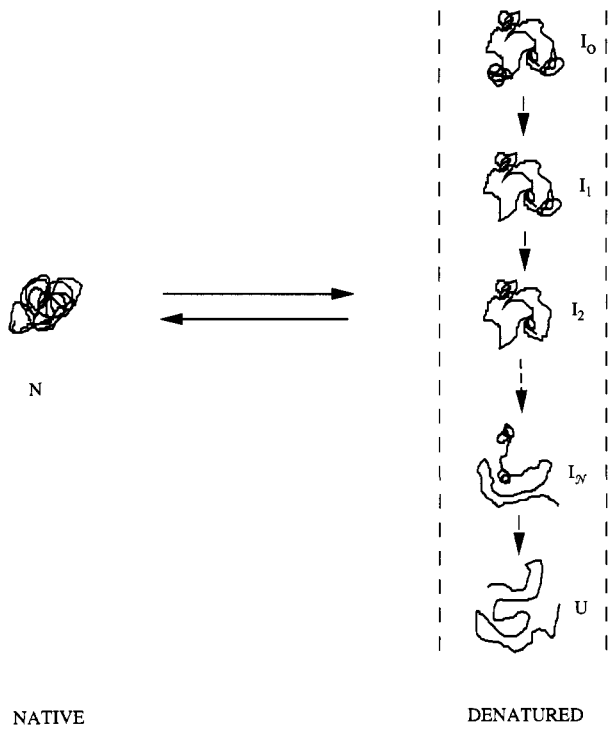

FIGURE 6: Schematic representation of the hierarchical cooperative (HC) model for protein folding/unfolding. The left column represents the unique native state conformation. The right column represents the ensemble of denatured states. By definition, all these states lack global cooperativity and are characterized by varying degrees of residual structure. At the top of the column is the first state that lacks global cooperativity (I_o), which is called the compact denatured state. At the bottom of the column is the completely unfolded state (U), which is structureless and fully solvated. The middle of the column comprises denatured states with varying degrees of residual structure. Thermal denaturation can be viewed as a transition from the native state to a particular distribution of denatured molecules. Under certain solvent conditions, the unfolded state is the predominant species and the transition approaches the classical two-state transition observed for many globular proteins. Under other conditions, the predominant species could be the compact denatured state. In this case, a highly cooperative transition will be observed between the native and the denatured state. Upon further heating of the protein sample, the center of the distribution will gradually shift toward the unfolded state, giving rise to a noncooperative transformation of the compact denatured state into the unfolded state.

& Murphy, 1991). For any given protein, the number and extent of the low-order structures will be a function of temperature and solvent conditions. Under certain conditions, low-order structures can be more stable than high-order structures and may serve as nucleation centers during the folding process. Conversely, during the unfolding process those elements will give rise to the appearance of residual structure once the long-range cooperative behavior is disrupted.

From a thermodynamic point of view, the global cooperativity that characterizes the native state is achieved when high-order structures have a higher stability than low-order structures. This is the situation that gives rise to the classical two-state folding/unfolding behavior. In this case, the breakdown of the native structure results in the complete unfolding of the protein. If, on the other hand, a subset of low-order structures have a higher stability than the native state, denaturation will not result in complete unfolding. Under those conditions, denaturation will produce an ensemble of conformations with different degrees of residual structure. This situation is illustrated in Figure 6. In this figure the right column represents the entire ensemble of conformations that define the denatured state. At the top of the column is the first state that lacks global cooperativity and at the bottom the completely unfolded state denoted by U. For convenience, the first state that lacks global cooperativity will be called the compact denatured state and will be denoted by I_o. Depending upon the solvent conditions and the temperature at which the transition occurs, the native state will denature to a particular distribution of denatured conformations. At low temperatures, the compact denatured state is the predominant species; however, as the temperature increases, the distribution progressively shifts toward conformations having fewer elements of residual structure until a temperature is reached in which the unfolded state is the predominant species. This model is called the hierarchical cooperative (HC) model (E. Freire, in preparation) and is summarized below.

Statistical Thermodynamic Formalism. The partition function for the hierarchical cooperative (HC) model is defined in the usual way as

$$Q = 1 + \sum_{i=I_o}^{U} \exp(-\Delta G_i/RT) \quad (1)$$

where ΔG_i is the Gibbs free energy of state i relative to that of the native state which is taken as the reference state, R is the gas constant, and T is the absolute temperature. According to the considerations illustrated in Figure 6, all the states included inside the summation sign lack global cooperativity. The mathematical implication of this feature of the HC model is that all terms inside the summation can be written as the product of two terms, one of which is the statistical weight of the first state that lacks global cooperativity (i.e., the compact denatured state).

If the statistical weight of the compact denatured or molten globule state (I_o) is factorized outside the summation sign, eq 1 becomes

$$Q = 1 + [\exp(-\Delta G_{I_o}/RT)] \sum_{i=I_o}^{U} \exp(-\Delta G'_i/RT) \quad (2a)$$

$$= 1 + [\exp(-\Delta G_{I_o}/RT)] Q_D \quad (2b)$$

where ΔG_{I_o} is the relative Gibbs free energy of the cooperative core of the molecule and Q_D is the internal partition function of the denatured state. The internal partition function for the denatured state can be written as

$$Q_D = \sum_{i=I_o}^{U} \exp(-\Delta G'_i/RT) \quad (3a)$$

$$Q_D = 1 + \sum_{i=I_1}^{U} \exp(-\Delta G'_i/RT) \quad (3b)$$

where the summation runs over all the conformations that define the denatured state. The Gibbs free energies $\Delta G'_i$ are referenced to the first state in the ensemble of denatured conformations (I_o) rather than the native state. It must be noted that if the fully unfolded state is the only denatured conformation $Q_D = 1$ and the complete folding/unfolding partition function reduces to the two-state partition function characteristic of many globular proteins (Freire & Biltonen, 1978; Privalov, 1979). As defined by eq 3, the number of accessible states having different amounts of residual structure is arbitrary; i.e., different proteins have a different number of residual structure elements. There are, however, two extreme situations. If the number is equal to 1, the total

folding/unfolding partition function will reduce to a three-state partition function:

$$Q = 1 + \exp(-\Delta G_{I_o}/RT) + \exp(-\Delta G_{I_o}/RT)\exp(-\Delta G'_U/RT) \quad (4a)$$

$$Q = 1 + \exp(-\Delta G_{I_o}/RT) + \exp(-\Delta G_U/RT) \quad (4b)$$

In this case, the compact denatured state transforms into the unfolded state in all-or-none fashion, and depending on the magnitude of the enthalpy difference between the unfolded and compact denatured state, this transformation might give rise to a significant heat absorption peak in the calorimeter. The experimental data corresponding to this situation as well as those corresponding to a few discrete states can be analyzed with the standard deconvolution equations for a multistate transition (Freire & Biltonen, 1978). In particular, the average excess enthalpy is given by

$$\langle \Delta H \rangle = \sum P_i \Delta H_i \quad (5)$$

where P_i is equal to the population of molecules in state i and is given by

$$P_i = \exp(-\Delta G_i/RT)/Q \quad (6)$$

The excess heat capacity function obtained by differential scanning calorimetry is simply the temperature derivative of the average excess enthalpy ($\langle \Delta C_p \rangle = \partial \langle \Delta H \rangle / \partial T$).

The second extreme is given by the case in which the denatured state comprises a large number of accessible states. In this case, the denatured state defines a continuum of substates separated by very small enthalpy differences. The transformation of the compact denatured state into the unfolded state proceeds gradually in a noncooperative fashion and with a minimal excess heat capacity. During this process, the enthalpy of the denatured state increases almost continuously as the distribution of states progressively shifts toward the unfolded state. In this case, the free energy difference between contiguous substates can be approximated by a differential (δG) and the partition function becomes

$$Q = 1 + [\exp(-\Delta G_{I_o}/RT)]\sum_{i=0}^{\mathcal{N}} \frac{\mathcal{N}!}{i!(\mathcal{N}-i)!}\exp(-i\delta G/RT) \quad (7a)$$

$$= 1 + [\exp(-\Delta G_{I_o}/RT)][1 + \exp(-\delta G/RT)]^{\mathcal{N}} \quad (7b)$$

In eq 7a, the summation runs over the total number of independent residual structure elements in the denatured state. The intrinsic free energy of a substate with i residual structural elements is simply $i\delta G$ since the free energy difference between contiguous substates is assumed to be the same. The combinatorial terms are equal to the number of substates having i residual structural elements out of a total of $\mathcal{N}$ elements. Since these terms correspond to the binomial expansion, eq 7a can be written in closed form as indicated in eq 7b. In this case, the average excess enthalpy function is equal to

$$\langle \Delta H \rangle = \frac{1}{Q}\{[\exp(-\Delta G_{I_o}/RT)][1 + \exp(-\delta G/RT)]^{\mathcal{N}}\Delta H_{I_o} + \mathcal{N}[\exp(-\Delta G_{I_o}/RT)][\exp(-\delta G/RT)][1 + \exp(-\delta G/RT)]^{\mathcal{N}-1}\delta H\} \quad (8)$$

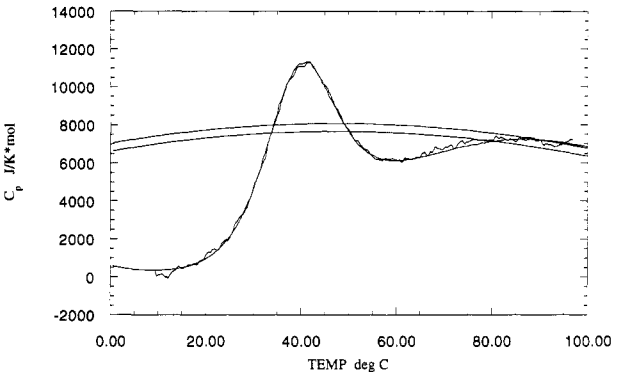

FIGURE 7: Temperature dependence of the excess heat capacity of aLA at pH 8, 500 mM Tris. In this figure, the experimental data and the theoretical curve calculated with the hierarchical cooperative model are shown. Also shown in the figure is the expected heat capacity change for the completely unfolded aLA calculated from its amino acid sequence (the double line indicates the uncertainty in the calculated values).

and, as before, the excess heat capacity function is the temperature derivative of $\langle \Delta H \rangle$. It must be noted that for $\mathcal{N} = 0$, the HC model (eqs 7 and 8) is equivalent to the two-state model, and for $\mathcal{N} = 1$, it corresponds exactly to the three-state model. For higher $\mathcal{N}$ values, the assumption that the free energy difference between contiguous substates is the same is the most conservative, given that the unfolding of the compact denatured state is a very broad process with no features revealing the presence of dissimilar states. It must be noted also that this assumption only affects the shape of the transition and not the overall magnitude of the thermodynamic parameters between the unfolded and the compact denatured state. Finally, from a phenomenological point of view, the parameter $\mathcal{N}$ is equal to the calorimetric to van't Hoff enthalpy ratio ($\Delta H_{cal}/\Delta H_{VH}$) for the unfolding of the compact denatured state.

ANALYSIS OF EXCESS HEAT CAPACITY FUNCTION

Apo-Lactalbumin at Neutral pH. The excess heat capacity function of aLA at pH 8 and a Tris concentration of 500 mM is shown in Figure 7. This function is characterized by two important features. First, there is a highly cooperative transition centered at 41 °C, and second, there is a gradual increase in the heat capacity function immediately following the first transition. As shown in the figure, the magnitude of the heat capacity after the first transition does not correspond to that of the unfolded state; however, it approaches that value at higher temperatures. From a qualitative point of view, these data indicate that the complete unfolding of aLA, under the conditions of these experiments, proceeds in two stages that mimic the sequence of events illustrated in Figure 6.

The excess heat capacity function of aLA was analyzed in terms of the HC model using non-linear least-squares analysis. This analysis was used to obtain the best estimates for the thermodynamic parameters that define the system. During the analysis, the magnitude of the total heat capacity change was allowed to vary within the experimental uncertainty limits in the determination of the absolute magnitude of the heat capacity (±600 J/K mol). Also, different temperature dependencies for the heat capacity changes were considered. The solid line in Figure 7 corresponds to the best fit of the data and is given by the parameters shown in Table 2.

The first observation that can be made is that, in all cases,

Table 2: Analysis of Heat Capacity Function of aLA (pH 8, 500 mM Tris) in Terms of Hierarchical Cooperative Model[a]

transition		A	B
N → I	$\Delta H(25)$	123 837 ± 13 000	111 104 ± 13 800
	$\Delta S(25)$	388.7 ± 0.9	357.4 ± 0.9
	$\Delta C_p(0)$	2 700 ± 630	3 728 ± 900
	A	22.2	0
	B	−0.235	0
I → U	$\mathcal{N}$	2.8 ± 0.5	1.94 ± 0.2
	$\Delta H(25)$	−70 182 ± 2 340	−38 673 ± 4 500
	$\Delta S(25)$	−229.1 ± 1	−127.5 ± 13
	$\Delta C_p(0)$	3 536 ± 350	2 880 ± 569
	A	22.2	0
	B	−0.235	0
	SSR	28.3	34.8

[a] ΔH's are in J/mol; ΔS's and ΔC_p's are in J/K mol. The coefficients A and B define the temperature dependence of the heat capacity changes ($\Delta C_p(T) = \Delta C_p(0) + AT + BT^2$). These coefficients were obtained from the temperature dependence of the heat capacity of the unfolded state and were not allowed to float during the fitting procedure. Columns A and B show the best parameter values assuming a temperature-dependent or a temperature-independent ΔC_p, respectively. For convenience, all thermodynamic parameters have been normalized per mole of protein rather than per mole of structural element.

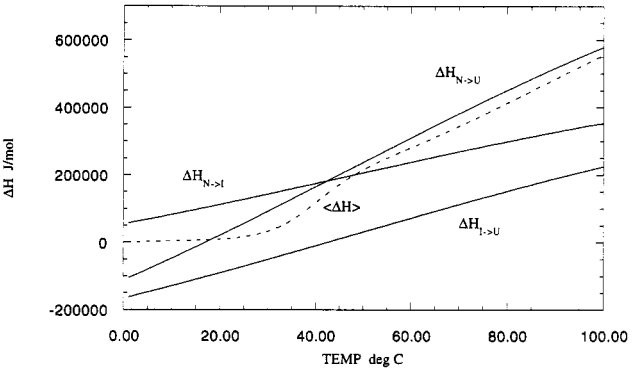

FIGURE 8: Temperature dependence of the enthalpy change for complete unfolding, the enthalpy change between the compact denatured and the native state, and the enthalpy change between the unfolded and compact denatured states (data for aLA at pH 8, 500 mM Tris). Also shown in the figure is the average excess enthalpy function ($\langle \Delta H \rangle$). Below 45 °C, the compact denatured state has a higher enthalpy than the unfolded state. Under those conditions, an increase in temperature cannot induced a transition to the unfolded state since the unfolded state has a lower enthalpy than the compact denatured state.

the best fit is obtained with a value of $\mathcal{N}$ between 2 and 3. This value is statistically larger than the values expected for a two-state or for a three-state transition, suggesting that the compact denatured state is not composed of a single cooperative unit. Most likely, this state is composed of residual structural elements that behave independently of each other, thus eliciting a poor cooperative behavior.

Figure 8 shows the temperature dependence of the enthalpy change for complete unfolding and the enthalpy change between the compact denatured and the native state. As shown in the figure, below 45 °C the compact denatured state has a higher enthalpy than the unfolded state. Under those conditions, an increase in temperature cannot induce a transition to the unfolded state since the unfolded state has a lower enthalpy than the compact denatured state. At higher temperatures, however, the unfolded state progressively becomes the high-enthalpy state since this state has a higher heat capacity than the compact denatured state. As the temperature increases, the average excess enthalpy of the system (i.e., the integral of the excess heat capacity function) approaches the enthalpy of the unfolded state; however, even

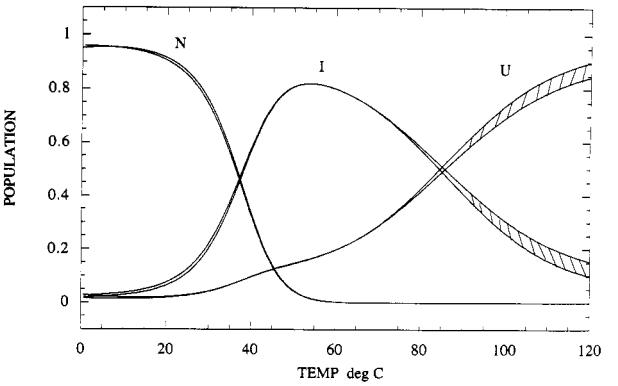

FIGURE 9: Temperature dependence of the population of molecules in the native state (N), unfolded state (U), and in the ensemble of denatured molecules excluding the unfolded state (I) (data for aLA at pH 8, 500 mM Tris). The double line represents the scattering resulting from the uncertainties in the calculated thermodynamic parameters.

at 100 °C not all the protein molecules are in the unfolded state, as shown in Figure 9.

At 45 °C, the heat capacity difference between the compact denatured state and the native state is close to 3.3 kJ/K mol, whereas that between the unfolded and the native state is close to 7.3 kJ/K mol. This result indicates that a significant fraction of the hydrophobic core of the protein is still present in the compact denatured state. The hydrophobic core disappears gradually upon increasing temperature as the distribution of denatured states shifts from the compact denatured to the unfolded state.

As shown in Figure 9, immediately after the main denaturation peak at 41 °C, about 80% of the protein molecules are in the compact denatured state while only 20% are in the unfolded state. As the temperature increases, the distribution of denatured states gradually shifts toward less structured states and the unfolded state progressively becomes the most significantly populated.

Lactalbumin at Acidic pH. The excess heat capacity function of holo-LA obtained at pH values ranging between 5.2 and 3.0 (Figure 1) was also analyzed in terms of the HC model. In all cases, the experimental curves could be accurately adjusted by this model using the parameters summarized in Table 3. Each curve was considered individually, so that any conformational effects of pH are implicitly included in the reported energetics. According to these parameters, at pH 5.2 the transition is close to a two-state transition, and thermal denaturation results in complete unfolding with no measurable population of compact denatured molecules. As the pH decreases and thermal denaturation occurs at lower temperatures, the transition results in a progressively higher proportion of compact denatured molecules. It must be noted also that the population of molecules in the compact denatured state is a function of both pH and temperature. At any given pH, the population of molecules in this state decreases upon increasing temperature, and at any given temperature, the population increases at lower pH values. This conclusion can be qualitatively appreciated by inspection of the absolute values of the heat capacity function at temperatures above the main transition.

As shown in Table 3, the enthalpy difference between the compact denatured state and the native state is to a large extent independent of pH, within the range studied. At 25 °C, the enthalpy difference between the compact denatured and the native state is on the order of 104.5 kJ/mol. The pH dependence of the transition temperature between the native

Table 3: Analysis of Heat Capacity Function of holo-LA As Function of pH in Terms of Hierarchical Cooperative Model[a]

	N → I_0			I_0 → U			N → U		
pH	$\Delta H(25)$	$\Delta S(25)$	$\Delta C_p(0)$	$\mathcal{N}$	$\Delta H(25)$	$\Delta S(25)$	$\Delta C_p(0)$	$\Delta H(25)$	$\Delta S(25)$	$\Delta C_p(0)$
5.2								−2 499	−66.67	7500
4.2	98 209	272.5	4370	0.8	−86 821	−278.3	2730	11 386	−5.8	7100
3.5	103 789	322.9	4310	1.6	−48 893	−167.0	2390	54 895	155.9	6700
3.2	109 658	358.3	4280	2.4	−19 976	−77.2	1990	89 677	281.1	6270
3.0	122 608	415.8	4000	2.2	−15 603	−33.1	1450	107 003	382.7	5450

[a] ΔH's are in J/mol; ΔS's and ΔC_p's are in J/K mol. In all cases the heat capacity for the complete unfolding of the native state was represented by the equation $\Delta C_p(T) = \Delta C_p(0) + 44.3T - 0.468T^2$, where T is in °C. The temperature dependence of the intermediate changes was weighted according to the magnitude of $\Delta C_p(0)$.

and compact denatured state is primarily entropic, as expected for situations in which the protonation enthalpies of the responsible groups is small (Privalov, 1979). The situation is different for the transition between the compact denatured state and the unfolded state. In this case, the enthalpy change at 25 °C is strongly dependent on pH, and it appears to increase monotonically as the pH decreases. This pH dependence is reflected in the overall enthalpy for complete unfolding which, at 25 °C, also increases as the pH decreases. This dependence, however, appears to be related to a pH-dependent decrease in ΔC_p (see Figure 1). In fact, when the enthalpies are extrapolated to 100 °C they assume similar values. The heat capacity difference between the unfolded and the native states decreases from about 7.5 kJ/K mol at pH 5.2 to about 6.3 kJ/K mol at pH 3.2. This is however a small decrease and requires further investigation regarding its exact dependence and molecular origin. The unfolded state may not be completely solvated at low pH, and an increase in the charge of the polypeptide chain below its isoelectric point may contribute negatively to the heat capacity.

As seen in Table 2, the value of $\mathcal{N}$ is essentially zero at pH 5.2, indicating that the transition conforms closely to a two-state transition. This is also illustrated in Figure 10, where the probability of each state has been plotted as a function of temperature. At pH 5.2, the compact denatured state never becomes populated, and the transition involves only the native and the unfolded states. At pH 4.2, the value of $\mathcal{N}$ is 0.8 and increases monotonically as the pH of the solution decreases. This result indicates that, at low pH values, the cooperativity of the unfolding transition of the compact denatured state becomes smaller, resulting in a barely noticeable excess heat capacity. The population of molecules in the compact denatured state increases at lower pH values. At pH 4.2, about 50% of the molecules is in the compact denatured state immediately after the transition; however, at pH 3.5 this percentage is close to 80% and at pH 3.0 it reaches about 100% of the protein molecules.

DISCUSSION

The unfolded state is not the highest enthalpy state of a protein under all conditions. For α-lactalbumin the compact denatured or molten globule state has a higher enthalpy than the unfolded state at temperatures below 45 °C. According to the LeChatelier principle, under those conditions a temperature increase cannot induce a transition from the compact denatured state to the unfolded state. Nevertheless, since the unfolded state has a higher heat capacity than the compact denatured state, the situation does not remain the same at all temperatures. As the temperature increases, the enthalpy of the unfolded state approaches and eventually surpasses the enthalpy of the compact denatured state. The unfolded state becomes the highest enthalpy state, and at that point it begins to be stabilized by a temperature increase. It should be noted that this conclusion does not depend on any model assumptions since it follows directly from the observed heat capacity increments for denaturation and unfolding.

The energetics of the different states of LA can be used to assess the type of interactions that stabilize the compact denatured state of the molecule. First is the heat capacity difference between states. It is known that the change in heat capacity associated with a change in protein conformation contains a positive contribution due to the exposure of apolar groups to water, a negative contribution due to the exposure of polar groups, and the overall magnitude of the change in heat capacity is proportional to the apolar and polar surface areas that become exposed (Murphy et al., 1992; Privalov & Makhatadze, 1992). Upon complete unfolding, LA exposes on the order of 6780 Å^2 of apolar surface and 4750 Å^2 of polar surface to the solvent (Haynie & Freire, 1993). These values are consistent with a heat capacity change on the order of 7.5 kJ/K mol as observed experimentally under conditions in which the protein undergoes complete unfolding (e.g., the experiments at pH 5.2). The heat capacity difference between the compact denatured state and the native state is on the order of 4 kJ/K mol for the experiments at acid pH and about 3 kJ/K mol for aLA at pH 8. Both figures are consistent with the presence of a significant hydrophobic core, approximately half the size of the one found in the native state.

Perhaps the most significant feature of LA and its compact denatured state is given by its enthalpy function: the total enthalpy of unfolding and its components, the enthalpy of denaturation, and the enthalpy of unfolding of the compact denatured state. It is known that the large positive change observed upon unfolding of the native structure of a protein is given primarily by the disruption of hydrogen bonds and van der Waals interactions (Privalov & Gill, 1988; Freire et al., 1992; Murphy & Freire, 1992; Makhatadze & Privalov, 1993). This positive enthalpy change is opposed by a negative enthalpy change that results from the hydration of those groups after their exposure to water (Privalov & Gill, 1988; Freire et al., 1992; Murphy & Freire, 1992; Makhatadze & Privalov, 1993). The total enthalpy of unfolding of LA increases with temperature; however, its extrapolated value at 110 °C is smaller than the value found for other globular proteins, in particular the structurally analogous protein lysozyme. Since the total heat capacity of unfolding is close to the expected value, one can assume that the lower enthalpy value is due to reduced van der Waals interactions and hydrogen bonding in the native state of LA. This assumption is supported by the known fact that LA is less rigid than lysozyme, judging by its faster hydrogen exchange kinetics, by the flexibility of the C terminal region in human LA (Acharya et al., 1991), and also by the larger absolute value of the heat capacity of its native state.

The most remarkable feature of the enthalpy changes for the individual stages of the unfolding process resides in their

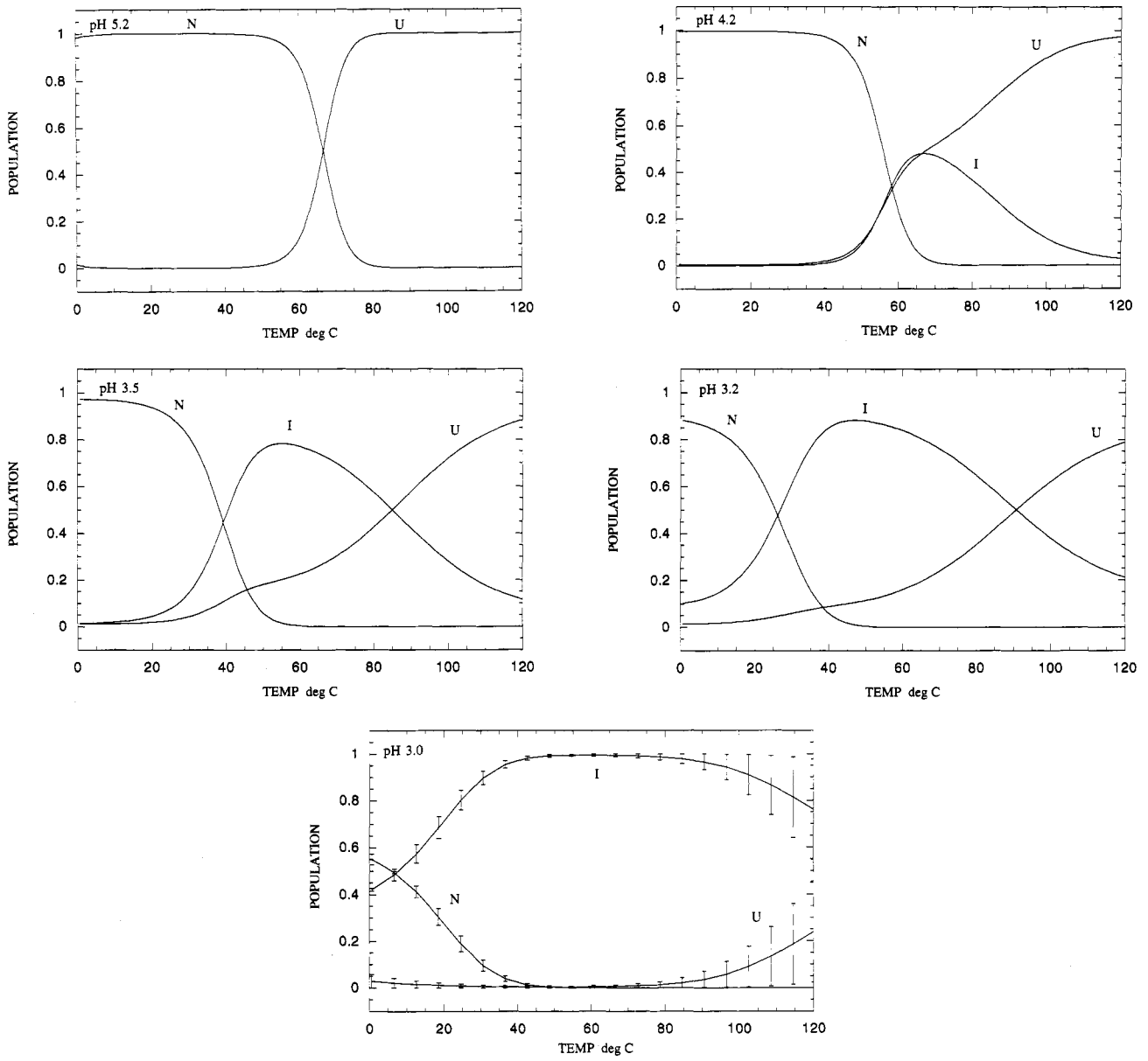

FIGURE 10: (Panels A–E) Temperature dependence of the population of molecules in the native state (N), in the unfolded state (U), and in the ensemble of denatured molecules excluding the unfolded state (I) for the data obtained at different pH values. The error bars are only shown for curve E since this case represents the situation with the largest errors.

temperature dependencies or heat capacities. As mentioned before, the heat capacity increments for the denaturation of the native state and for the unfolding of the compact denatured state are of the same order of magnitude, indicating that about half of the hydrophobic core is preserved in the compact denatured state. If the enthalpy values are compared at 100 °C (see Figure 8), a temperature at which the hydration enthalpy of nonpolar groups is close to zero, it follows that the enthalpy difference between the compact denatured state and the native state is higher than that between the unfolded state and the compact denatured state. These values indicate that a larger number of van der Waals contacts are disrupted in the transition from the native to the compact denatured state, implying that the compact denatured state has a much looser structure than the native state as confirmed by other experimental evidence (Kuwajima, 1989; Ptitsyn, 1992). It should be noted that a looser structure does not necessarily means that its internal groups are accessible to water. This structure might be compact enough to exclude water penetration to some extent, but the averge distance between groups could be longer than in the tightly packed interior of the native protein; i.e., this structure resembles a molten globule and not the highly packed crystal-like structure of the native state.

At 100 °C, the enthalpy of the unfolded state is larger than that of the compact denatured state, since the former lacks all van der Waals and hydrogen-bonding interactions while a substantial number are still present in the compact denatured state. At temperatures below 45 °C, however, the enthalpy of the compact denatured state is higher than that of the unfolded state. The reason for this effect is the persistence of a sizable hydrophobic core in the compact denatured state. As a result, a significant number of residues are not exposed to water in the compact denatured state, resulting in a negative enthalpy contribution smaller than in the unfolded state. Since the magnitude of the negative enthalpy contribution increases upon decreasing temperature and with a faster rate for the unfolded state, it follows that at temperatures lower than a certain temperature the enthalpy of the compact denatured state will be higher than that of the unfolded state. If the compact denatured state had a smaller hydrophobic core, a

significant number of apolar residues would have been exposed to water, resulting in the inability to offset the large positive enthalpy of the unfolded state within the accessible temperature range. These results suggest that a hydrophobic core larger than a critical size might be required for the stabilization of the compact denatured state. In this respect, it should be noted that LA has a higher proportion of apolar residues than lysozyme (59% vs 56% in terms of buried areas), which exists in the completely unfolded state under conditions in which LA is in the compact denatured state.

The temperature at which the enthalpy difference between the unfolded and the compact denatured state is zero depends on the solvent conditions, ranging anywhere between 40 and 60 °C (see Table 3). Below this temperature, the difference is negative and above this temperature it is positive; however, in the neighborhood of the inversion temperature the absolute values of the enthalpy are rather small. This fact has substantially complicated the experimental determination of the energetics of the compact denatured or molten globule state and, in the past, has led to the conclusion that it is enthalpically equivalent to the unfolded state (Pfeil et al., 1986; Yutani et al., 1992).

The transition from the compact denatured state to the unfolded state is rather noncooperative. In fact, under conditions in which the compact denatured state is significantly populated, the best fitting parameters are consistent with a $\mathcal{N}$ value on the order of 2–3. This range of values is significantly larger than the value of 1 expected for a three-state transition. The net effect of this lack of cooperativity is that the transition proceeds with little transition excess heat capacity (Haynie & Freire, 1993) approaching the macroscopic behavior of a transition characterized by no discontinuity in the first derivative of the thermodynamic potential, i.e., it resembles a higher-order phase transition. It must be remembered that the heat capacity function is composed of two terms: the transition excess heat capacity responsible for the characteristic "bell-shaped" transition peaks associated with protein denaturation and the "S-shaped" baseline shift due to the existence of a positive ΔC_p. This latter term is an ensemble average directly proportional to the population of molecules in the denatured and unfolded states. Due to the absence of a significant transition excess heat capacity, the measured heat capacity does not exhibit a clear peak and seems to monotonically approach the heat capacity of the unfolded state as the temperature increases (in some cases, the excess heat capacity function may look like an S-shaped curve). Throughout this gradual increase in heat capacity, the enthalpy of the system increases as the distribution of denatured molecules progressively shifts toward the unfolded state. During this process, it is possible for some system parameters to exhibit transitions that occur in a narrower temperature range (Lifshitz et al., 1978; Dill & Shortle, 1991). This is also true for physical observables that reflect the population of only one or a few states rather than the entire ensemble average as is the case for the excess enthalpy function. These transitions occur within narrow limits of the excess enthalpy function and, as such, they are not accompanied by a significant enthalpy change. For example, as the degree of unfolding of the denatured state increases, the protein molecule undergoes a transition from a collapsed conformation to an extended one. This change of state is not necessarily a linear function of the degree of unfolding and might occur within a small domain of the excess enthalpy and, as such, be accompanied by a relatively small enthalpy change. In the limit in which this enthalpy change approaches zero, it can be regarded as a second or higher order phase transition.

Recent NMR results on the acid-denatured state of guinea pig LA (Chyan et al., 1993) suggest that the compact denatured state is highly heterogeneous in terms of the stability and the specificity of backbone and side-chain interactions. Also, Ewbank and Creighton (1993) have concluded from a study of specific three- and two-disulfide folding intermediates that the molten globule state of LA is devoid of cooperative or specific tertiary interactions. These observations are in agreement with the $\mathcal{N}$ values obtained from our analysis of the heat capacity function. It must be noted, however, that the absence of a cooperative compact denatured–unfolded state transition is not a general feature of the model. Other proteins might have a smaller $\mathcal{N}$ value and exhibit a clearly visible heat capacity peak associated with the unfolding of the compact denatured state. In fact the molten globule states of the retinol binding protein (Bychkova et al., 1992) and equine lysozyme (C. M. Dobson, personal communication) undergo cooperative thermal unfolding transitions.

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