Calorimetric Determination of the Energetics of the Molten Globule Intermediate in Protein Folding: Apo-α-lactalbumin†

Dong Xie, Vinod Bhakuni, and Ernesto Freire*
Department of Biology and Biocalorimetry Center, The Johns Hopkins University, Baltimore, Maryland 21218
Received June 3, 1991; Revised Manuscript Received September 3, 1991

ABSTRACT: High-sensitivity differential scanning calorimetry has been used to characterize the energetics of the molten globule state of apo-α-lactalbumin. This characterization has been possible by performing temperature scans at different guanidine hydrochloride (GuHCl) concentrations in order to experimentally define the temperature–GuHCl stability surface of the protein. Multidimensional analysis of the heat capacity surface has allowed simultaneous resolution of the energetics of the unfolded and molten globule states. These experiments indicate that the intrinsic enthalpy difference (i.e., excluding additional contributions such as those arising from differential GuHCl binding) between the unfolded and native states is 31.8 kcal/mol at 25 °C whereas that of the molten globule and native states is only 7.7 kcal/mol. At the same temperature, the entropy changes are 99.2 and 23.7 cal/K·mol and the heat capacity changes are 1821 and 326 cal/K·mol, respectively. Analysis of the thermodynamic data indicates that in passing from the native to the molten globule state only ~19% of the hydrogen bonds are broken. In addition, the magnitude of ΔC_p for the molten globule suggests that water does not largely penetrate into the interior of the molten globule, implying that significant hydrophobic interactions are still present in this state. These parameters provide precise energetic constraints to the allowed structural conformations of the molten globule.

Protein folding/unfolding is generally a highly cooperative process in which only the completely folded and unfolded states become significantly populated. Under certain circumstances, however, several proteins have been shown to exhibit a folding intermediate known as a "molten globule" (Kim & Baldwin, 1982, 1990; Kuwajima, 1989; Baum et al., 1989; Hughson et al., 1990; Christensen & Pain, 1991; Ewbank & Creighton, 1991). The molten globule state of apo-α-lactalbumin (MW = 14 200) is the most extensively characterized and has been shown to be stable at low ionic strength, low pH, or in the presence of intermediate concentrations of denaturants (Kuwajima et al., 1976, 1989; Semisotnov et al., 1987; Baum et al., 1989; Jeng et al., 1990). The molten globule intermediates found for different proteins appear to have some common features, primarily a native-like secondary structure, a high degree of compactness, and a disrupted tertiary structure (Ohgushi & Sugai, 1983; Ptitsyn, 1987; Kuwajima, 1989). The CD spectrum of the molten globule state of apo-α-lactalbumin is similar to that of the native protein in the far-UV region but nearly absent in the near-UV region, consistent with an almost intact secondary structure but disrupted tertiary structure (Dolgikh et al., 1985; Kuwajima, 1989). The molten globule state has also been shown to exhibit a rotational correlation time and viscosity close to that of the native state, indicating that the molten globule is highly compact (Dolgikh et al., 1981). Ewbank and Creighton (1991) have recently shown that the molten globule state is compatible with a variety of disulfide bonding pairings, suggesting the absence of many specific tertiary structure interactions.

Even though a significant number of experimental approaches have been used to characterize the molten globule state, until now the energetics of this state have not been experimentally addressed. To the best of our knowledge, this paper presents the first direct differential scanning calorimetric characterization of the energetics of the molten globule state of a protein.

† Supported by Grants RR04328 and NS24520 from the National Institutes of Health.

Material and Methods

Proteins. Bovine apo-α-lactalbumin was purchased from Sigma (St. Louis, MO) and reconstituted in 10 mM borate buffer, 0.2 M NaCl, and 1 mM EDTA, pH 8.0. The protein solution was dialyzed overnight against 4000 volumes of the same buffer at 4 °C. Protein concentrations were determined spectrophotometrically using a value of $E_{1\%}$ of 20.1 at 280 nm for a 1-cm path length (Kornman & Andreotti, 1964). Hen egg white lysozyme was also purchased from Sigma and dialyzed against 15 mM acetate and 0.2 M NaCl, pH 4.5. Lysozyme concentrations were determined spectrophotometrically using a $E_{1\%}$ value of 26.9 at 280 nm (Pfeil & Privalov, 1975). GuHCl stock solutions were prepared in the appropriate protein buffers, the pH was checked and adjusted, and the concentration was determined by refractive index measurements (Pace, 1986; Ramsay & Freire, 1990). The purity of the proteins was checked by SDS–polyacrylamide gel electrophoresis.

Differential Scanning Calorimetry. All calorimetric scans were performed with a Microcal MC-2 differential scanning calorimeter. The calorimetric unit was interfaced to an IBM PC microcomputer using an A/D converter board (Data Translation DT-2801) for automatic data collection and analysis. The samples were degassed for 15 min at room temperature prior to being scanned at rates of 60 °C/h. On occasion, scans were also run at 90 °C/h to check the absence of kinetic effects. The protein concentration for these experiments was 3–4 mg/mL. Excess heat capacity functions were obtained after baseline subtraction, as described previously (Freire & Biltonen, 1978; Freire, 1989; Ramsay & Freire, 1990).

Data Analysis. All data analysis was performed on a Hewlett-Packard 835 Turbo SRX superminicomputer. Nonlinear least-squares analysis was performed by using the Marquardt (Marquardt, 1963) and the Simplex minimization algorithms (Nelder & Mead, 1965) using software for multidimensional analysis (Ramsay & Freire, 1990) developed in this laboratory. This software implements the linkage between the folding/unfolding equations and the denaturant

0006-2960/91/0430-10673$02.50/0

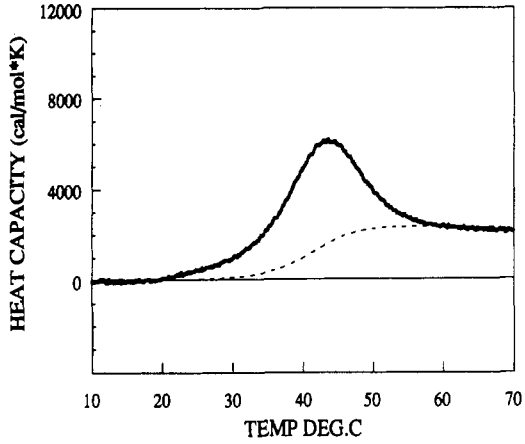


FIGURE 1: Heat capacity function versus temperature for apo-α-lactalbumin in 10 mM borate buffer, 0.2 M NaCl, and 1 mM EDTA, pH 8.0. The calorimetric scan was performed with a protein concentration of 3 mg/mL at a scanning rate of 60 °C/h. The reversibility of this and all the scans in this paper was better than 95%. The dotted line represents the characteristic sigmoidal baseline associated with proteins exhibiting a ΔC_p for the transition (see the text for details). The excess heat capacity function is obtained after subtracting the baseline from the heat capacity function.

binding equations, thus permitting a global analysis of the calorimetric scans as a function of several independent variables. By analyzing simultaneously the heat capacity surface defined along the temperature and denaturant concentration axis, the analysis permits the determination of a unique set of thermodynamic parameters for the entire set of protein folding/unfolding data. Error analysis of the fitted parameters was performed with the program NONLIN developed by Dr. Michael Johnson at the University of Virginia. This program implements a modified Gauss–Newton nonlinear least-squares algorithm for the determination of the most probable model parameter values and estimates nonlinear asymmetric joint confidence intervals for all variable model parameters by an approximate nonlinear support plane method (Johnson, 1983; Johnson & Frasier, 1985). In addition to the intrinsic fitting confidence intervals, the reported errors in this paper include potential errors arising from protein concentration determinations. To include this source of error in the data analysis, a simulated ±5% error in concentration was introduced at random into each curve and the global analysis performed again.

RESULTS

Differential Scanning Calorimetry. The heat capacity function of apo-α-lactalbumin measured at pH 8.0 and 0.2 M NaCl is shown in Figure 1. The transition is fully reversible as demonstrated by repeated scans of the same sample. As shown in the figure, the thermal unfolding of apo-α-lactalbumin is characterized by a single peak centered at 43.26 °C. Analysis of the excess heat capacity function obtained after baseline subtraction as indicated in the figure yields a calorimetric enthalpy (ΔH_{cal}) of 66 ± 3 kcal/mol and a van't Hoff enthalpy (ΔH_{vH}) of 58 ± 2 kcal/mol. Under these conditions, the transition is characterized by a $\Delta H_{vH}/\Delta H_{cal}$ ratio of 0.88, which is slightly, but significantly, smaller than 1. ΔH_{cal} is equal to the area under the curve, and ΔH_{vH} is obtained with the standard formula

$$\Delta H_{vH} = 4RT_m^2 C_{p,max}/\Delta H_{cal}$$

where $C_{p,max}$ is the maximum of the excess heat capacity function, T_m is the transition temperature defined as the temperature location of $C_{p,max}$, and R is the gas constant. The

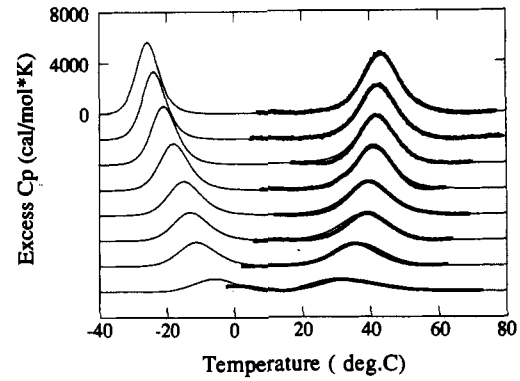


FIGURE 2: Excess heat capacity function versus temperature for apo-α-lactalbumin at increasing GuHCl concentrations. From top to bottom the concentrations are 0, 0.2, 0.35, 0.5, 0.7, 0.85, 1.0, and 1.5 M. The dots are the experimental data, and the solid lines are the theoretical curves predicted by the three-state molten globule model in Figure 4 and the parameters in Table I. For illustration purposes, the predicted cold denaturation curves have been included in the figure even though they are beyond our experimental limits. The curves have been shifted in the vertical axis for presentation purposes.

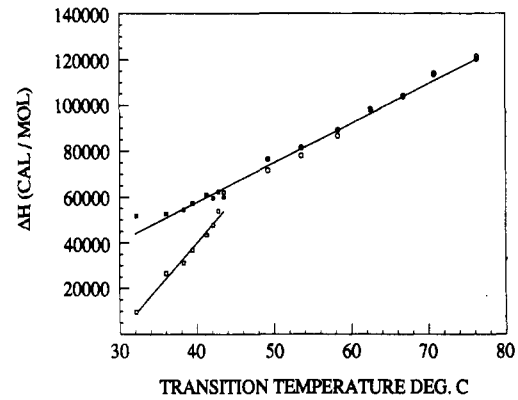


FIGURE 3: Temperature dependence of the calorimetric (open symbols) and van't Hoff enthalpy changes (solid symbols) associated with the thermal unfolding of apo-α-lactalbumin (squares) and hen egg white lysozyme (circles).

thermal unfolding of apo-α-lactalbumin is characterized by a ΔC_p of 1.9 kcal/K·mol. These values are in excellent agreement with the T_m of 43.5 °C, the ΔH_{cal} of 65 kcal/mol, and the ΔC_p of 1.5 kcal/K·mol previously obtained by Pfeil and Sadowski (1985).

Figure 2 shows the excess heat capacity function of apo-α-lactalbumin obtained at increasing concentrations of GuHCl ranging from 0 to 1.5 M. Baselines were subtracted as indicated in Figure 1 and under Material and Methods. The addition of GuHCl destabilizes the native conformation as seen by a decrease in T_m at increasing GuHCl concentrations. The enthalpy change for the transition also decreases at higher GuHCl concentrations; however, the measured decrease in the calorimetric enthalpy is much larger than the decrease expected from the ΔC_p associated with the transition. This point is illustrated in Figure 3, where the calorimetric and van't Hoff enthalpies for apo-α-lactalbumin obtained at different GuHCl concentrations have been plotted as a function of temperature. For comparison, the results obtained with the structurally homologous protein lysozyme are also shown in the figure. It is clear from this figure that the observed decrease in the calorimetric enthalpy of apo-α-lactalbumin is much larger than the decrease in the van't Hoff enthalpy and much larger than the observed change for lysozyme. Also, in this latter case the calorimetric and van't Hoff enthalpies are nearly equal to each other at all GuHCl concentrations. On the contrary,

for apo-α-lactalbumin, the van't Hoff to calorimetric enthalpy ratio increases upon increasing GuHCl concentrations due to the large decrease in ΔH_{cal}.

At increasing GuHCl concentrations (see Figure 3), the $\Delta H_{vH}/\Delta H_{cal}$ ratio for apo-α-lactalbumin increases monotonically to values larger than 1. For lysozyme, the $\Delta H_{vH}/\Delta H_{cal}$ ratio remains approximately equal to 1 at all GuHCl concentrations studied (0–3 M), indicating that under these conditions the thermal unfolding of this protein conforms closely to the two-state mechanism. As shown in Figure 3, the increase in the $\Delta H_{vH}/\Delta H_{cal}$ ratio occurs due to a decrease in the measured calorimetric enthalpy rather than to an increase in the van't Hoff enthalpy, arguing against protein oligomerization as a possible explanation for this behavior. In addition, experiments performed at different protein concentrations (1–10 mg/mL) yielded identical results. The calorimetric data are consistent with the notion that the addition of GuHCl shifts an increasing fraction of protein molecules to a state which appears not to undergo a cooperative or a significantly enthalpic unfolding transition, being therefore beyond the limits of calorimetric detection. Under those conditions, the observed calorimetric peak contains predominantly the contribution from the fraction of molecules still in the native state. Since the data are normalized to the total protein concentration, this behavior results in a reduced calorimetric enthalpy for the observed transition peak. This is the behavior expected for the molten globule state (Kuwajima, 1989).

In order to test the above idea, calorimetric experiments at different GuHCl concentrations were performed in the presence of excess Ca^{2+} (40 mM) (data not shown). Under these conditions, formation of the molten globule state is suppressed even at high GuHCl concentrations (Ikeguchi et al., 1986). In the presence of 40 mM Ca^{2+}, the thermal unfolding of α-lactalbumin was centered at 71.3 °C and characterized by a ΔH_{cal} of 80 kcal/mol, a ΔC_p of 1.6 kcal/K·mol, and a $\Delta H_{vH}/\Delta H_{cal}$ of 1.05. This ratio remains very close to 1 (1.07 ± 0.03) even at the highest GuHCl concentration studied (1 M) in the presence of Ca^{2+}. This result is consistent with previous studies indicating that under conditions of excess Ca^{2+} the unfolding transition closely conforms to the two-state mechanism even in the presence of GuHCl (Ikeguchi et al., 1986). On the contrary, at 1 M GuHCl, the $\Delta H_{vH}/\Delta H_{cal}$ for apo-α-lactalbumin is already equal to 2.2 and deviates very significantly from the two-state mechanism.

For apo-α-lactalbumin, the apparent ΔC_p calculated from the temperature dependence of the van't Hoff enthalpy (1.9 kcal/K·mol) obtained at different GuHCl concentrations is similar to the one determined directly (Figure 1), whereas that obtained from the calorimetric enthalpy is about 2.2 times larger (4.15 kcal/K·mol). For lysozyme, the ΔC_p values determined directly (data not shown) or from the temperature dependence of the van't Hoff and calorimetric enthalpies are all equal (1.8 kcal/K·mol).

Thermodynamic Analysis. The calorimetric data presented above indicate that, upon increasing the concentration of GuHCl, an increasing number of apo-α-lactalbumin molecules are shifted to a state that contributes significantly less to the unfolding transition, thus triggering a significant decrease in the calorimetric enthalpy. This conclusion is consistent with the CD data reported by Kuwajima (1977) indicating that the presence of moderate GuHCl concentrations, such as those studied here, induces the formation of the molten globule state. The view that emerges from these experiments is that, in the absence of GuHCl, the molten globule state is not largely

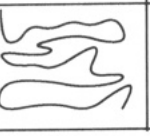
INDEX	STATE	FREE ENERGY	STAT. WEIGHT
F		0	1
A		$\Delta G^{o}_{A} - \Delta n_A RT\ln(1 + K_b X)$	$K^{o}_{A}(1 + K_b X)^{\Delta n_A}$
U		$\Delta G^{o}_{U} - \Delta n_U RT\ln(1 + K_b X)$	$K^{o}_{U}(1 + K_b X)^{\Delta n_U}$

FIGURE 4: Schematic representation of the states, free energies, and statistical weights associated with the folding/unfolding equilibrium of apo-α-lactalbumin. The state F is the folded, native state (taken as the reference state in this paper), the state A is the molten globule, and the state U is the unfolded state. $\Delta G°_A$, $K°_A$ and $\Delta G°_U$, $K°_U$ are the Gibbs free energies and stability constants of the A and U states in the absence of denaturant. X is the activity of the denaturant (GuHCl or urea), K_b is the binding constant of the denaturant, and Δn_A and Δn_U are the differences in the number of binding sites between the molten globule and folded states and the unfolded and folded states, respectively.

populated at any temperature. At increasing GuHCl concentrations, the molten globule state becomes progressively populated even at temperatures below the transition region. Under those conditions, the transition is characterized by the presence of three significantly populated states, the native or folded state, the molten globule state, and the unfolded state. The calorimetric data in Figure 2 permit a rigorous evaluation of the thermodynamic transition mechanism.

Figure 4 schematically represents the three states associated with the apo-α-lactalbumin transition as well as their free energies and statistical weights. According to those definitions, the folding/unfolding partition function can be written as

$$Q = 1 + K°_A(1 + K_b a_{GuHCl})^{\Delta n_A} + K°_U(1 + K_b a_{GuHCl})^{\Delta n_U} \quad (1)$$

where $K°_A$ and $K°_U$ are the stability constants of the A and U states in the absence of GuHCl, K_b is the binding constant of GuHCl, and Δn_A and Δn_U are the difference in the number of binding sites between the molten globule and folded states and the unfolded and folded states, respectively. These parameters are defined in Figure 4. The activity of GuHCl (a_{GuHCl}) was calculated using the mean ion activity coefficients published by Pace (1986) using the standard formula $a_{GuHCl} = (a_{\pm})^2$. The population of folded (P_F), molten globule (P_A), and unfolded states (P_U) are given by

$$P_F = \frac{1}{Q} \quad (2a)$$

$$P_A = \frac{K°_A(1 + K_b a_{GuHCl})^{\Delta n_A}}{Q} \quad (2b)$$

$$P_U = \frac{K°_U(1 + K_b a_{GuHCl})^{\Delta n_U}}{Q} \quad (2c)$$

The average excess enthalpy is

$$\langle \Delta H \rangle = P_A \Delta H_A + P_U \Delta H_U \quad (3)$$

where

$$\Delta H_A = \Delta H°_A + \Delta n_A \Delta H_b \frac{K_b a_{GuHCl}}{(1 + K_b a_{GuHCl})} \quad (4a)$$

$$\Delta H_U = \Delta H°_U + \Delta n_U \Delta H_b \frac{K_b a_{GuHCl}}{(1 + K_b a_{GuHCl})} \quad (4b)$$

Table I: Folding/Unfolding Energetics of Apo-α-lactalbumin[a]

state	$\Delta H°$ (25) (kcal/mol)	$\Delta S°$ (25) (cal/K·mol)	$\Delta C_p°$ (cal/K·mol)	Δn	ΔH_b (kcal/mol)	ΔS_b (cal/K·mol)
A	7.7 ± 0.1	23.7 ± 0.2	326 ± 2	18 ± 0.5	−2.62 ± 0.03	−9.23 ± 0.08
U	31.8 ± 0.1	99.2 ± 0.4	1821 ± 1	26 ± 0.7	−2.62 ± 0.03	−9.23 ± 0.08

[a] The values in the table correspond to pH 8.0 and 0.2 M NaCl. The quoted errors are the estimated uncertainties corresponding to one standard deviation. They include uncertainties in concentration determination as well as the correlated uncertainties in the model parameters estimated by NONLIN (see the text for details).

where ΔH_b is the binding enthalpy of GuHCl. $\Delta H°_A$ and $\Delta H°_U$ are the intrinsic enthalpy changes for the molten globule and the unfolded state, respectively. ΔH_A and ΔH_U are the observed apparent enthalpy changes for the molten globule and the unfolded state, respectively. The remaining terms in eqs 2–4 are defined in Figure 4. The heat capacity measured by differential scanning calorimetry is the temperature derivative of the excess enthalpy function

$$C_p = \frac{\partial \langle \Delta H \rangle}{\partial T} \tag{5}$$

The above equations were used to perform a global nonlinear least-squares analysis of the entire family of calorimetric data as a function of temperature and GuHCl concentration. The results of the analysis are summarized in Table I. The solid lines in Figure 2 are the theoretical excess heat capacities predicted by the parameters in Table I. For illustration purposes, the predicted cold denaturation portions of the curves have also been plotted in the figure even though those temperature regions are outside our experimentally accessible range. The cold denaturation of apo-α-lactalbumin has been experimentally demonstrated before by circular dichroism (Kuwajima, 1977). For comparison, the same analysis was performed assuming a two-state transition mechanism. This model yielded curves that adjusted poorly to the data as indicated by the variance of the fit, the *F*-statistics for the two-models, and the presence of systematic deviations in the residuals.

The thermodynamic values in Table I completely describe the folding/unfolding equilibrium as a function of temperature and GuHCl concentration. As mentioned above, the thermodynamic parameters in the absence of GuHCl are similar to those observed before by Pfeil and Sadowski (1985). The thermodynamic parameters for the binding of GuHCl agree with recent measurements by Makhatadze and Privalov (personal communication), also indicating a binding enthalpy of −2.6 kcal/mol and a binding constant of 0.6 − 0.7 at 25 °C. Also, Okuda and Sugai (1977) have previously estimated values of 23 and 10 for Δn_U and Δn_A, respectively. The excellent agreement between those parameters that have been measured independently with those obtained from the multidimensional analysis provides additional support to the validity of the approach used in this investigation. It should also be noted that the confidence intervals of the fitted parameters obtained from the global analysis are significantly better than those that can be obtained from the individual analysis of each curve. This is due to a more robustly defined variance space resulting from the introduction of a second independent variable.

Figure 5 shows the population of states as a function of temperature and GuHCl. At zero GuHCl and below the transition temperature region, the molten globule never reaches more than 16% of the total population of molecules. During the transition, however, it reaches approximately 30% of the total population. At intermediate GuHCl concentrations (1–1.5 M), the molten globule is maximally populated, reaching a total of 83% at 10 °C and 1 M GuHCl. At higher GuHCl concentrations (>2 M), the unfolded state becomes the most significantly populated state at all temperatures. These results agree with CD measurements obtained as a function of GuHCl concentration (Ikeguchi et al., 1986). According to their CD measurements, at 25 °C and 1.5 M GuHCl the molten globule population is approximately 47%. The population predicted by the parameters in Table I is 42%.

Discussion

The molten globule state of apo-α-lactalbumin as well as other proteins is thought to be a compact conformation with a secondary structure content similar to that of the native state but a poorly defined tertiary structure. Recently, it has been shown that the molten globule is compatible with a variety of disulfide bond pairings, suggesting that it does not exist in a single preferred conformation (Ewbank & Creighton, 1991). The experiments presented here provide rigorous energetic constraints to the possible conformations of the molten globule.

It has been shown that the enthalpy change associated with protein thermal unfolding contains primarily contributions arising from the solvent exposure of buried apolar groups in the protein as well as the rupture of secondary and tertiary structure hydrogen bonds (Privalov & Gill, 1988). Recently, the existence of convergence temperatures for the enthalpy and entropy changes of globular proteins has been observed (Baldwin, 1986; Privalov & Gill, 1988). It has been argued that at these temperatures, $T_H^* \cong 100$ °C and $T_S^* \cong 112$ °C, respectively, the hydrophobic contributions to the enthalpy and entropy change are zero (Murphy & Gill, 1990, 1991). At 100 °C the measured enthalpy change is primarily due to the disruption of hydrogen bonds. At this temperature, ΔH_A is 32 kcal/mol and ΔH_U is 169 kcal/mol, suggesting that the passage of the native state to the molten globule involves the rupture of only ~19% of all the hydrogen bonds in the molecule. Dolgikh et al. (1985) have shown spectroscopically that the α-helix and β-sheet content of the native and molten globule states do not differ by more than 10% and 5%, respectively. Analysis of the crystallographic structure of α-lactalbumin indicates that it has a total of 84 hydrogen bonds, of which 69 are secondary structure bonds and 15 are tertiary structure hydrogen bonds. It is interesting to note that the number of tertiary structure hydrogen bonds is similar to the number of hydrogen bonds estimated to be broken in the molten globule.

The transition enthalpies in Table I are consistent with the notion that in the molten globule state the secondary structure is almost intact but the tertiary structure is highly disrupted. Also, it is important to note that these enthalpy values are intrinsic enthalpy values. The experimentally measured values contain additional contributions particularly those arising from the differential GuHCl binding. For example, at 25 °C and 1.5 M GuHCl the effective apparent enthalpy difference between the molten globule and the unfolded state is only ~10 kcal/mol, i.e., a value too small to yield a sharp, highly "cooperative" transition. The absence of such cooperative

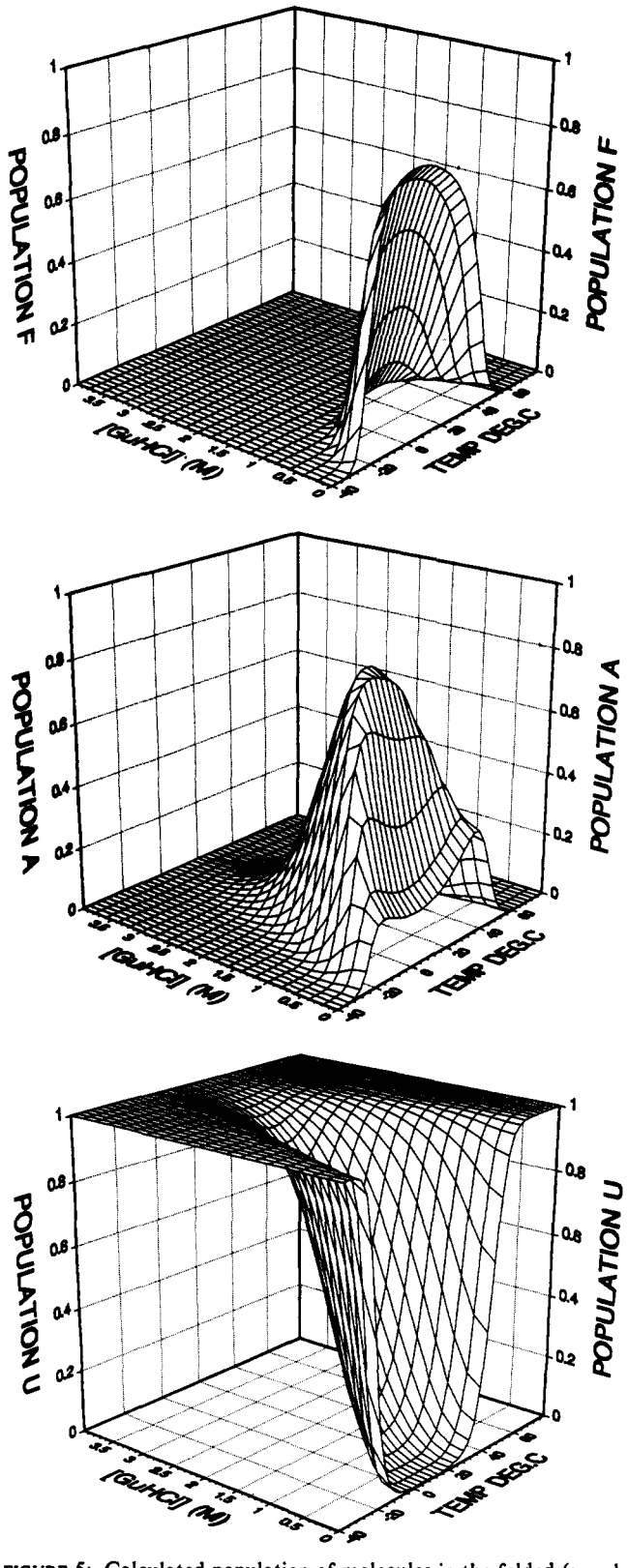


FIGURE 5: Calculated population of molecules in the folded (panel A, top), molten globule (panel B, middle), and unfolded (panel C, bottom) states of apo-α-lactalbumin as a function of temperature and GuHCl concentration.

unfolding of the molten globule state has been observed before by other authors (Kuwajima, 1989). A somewhat similar situation is expected to occur with the molten globule state obtained at very low pH. In this case, the transition from the molten globule to the unfolded state is coupled to the protonation of several ionizable groups (Kuwajima et al., 1981) that contribute to the observed enthalpy.

The heat capacity change associated with the unfolding of proteins contains a large positive contribution due to the exposure of previously buried hydrophobic groups to water and a smaller negative contribution associated with the rupture of hydrogen bonds (Murphy & Gill, 1990, 1991; Privalov & Makhatadze, 1990). On the basis of solid model compound studies, Murphy and Gill (1990, 1991) have estimated these contributions as 6.69 cal/K·mol of apolar hydrogen exposed to water and −14.3 cal/K·mol of hydrogen bond and have shown that these values accurately predict the values obtained for globular proteins. These values also predict the observed ΔC_p for apo-α-lactalbumin with high accuracy (1.93 kcal/K·mol compared to the experimental value of 1.8 kcal/K·mol). Using this approach, we estimate that approximately 16–20% of the total number of buried apolar groups become exposed to water in the molten globule state.

The picture that emerges from these studies is that the molten globule state is energetically compatible with an almost intact secondary structure in agreement with CD, infrared, and NMR data (Dolgikh et al., 1985; Baum et al., 1989). However, the magnitude of the observed ΔC_p is not abnormally high, suggesting that the molten globule exposes only a small fraction of the total number of buried apolar groups. This observation indicates that water does not significantly penetrate into the interior of the molten globule and that, therefore, the hydrophobic interactions within the molten globule state are still substantial. This conclusion is consistent with the view expressed recently by Kuwajima that the molten globule "*may be characterized as a nonspecific assembly of secondary structure segments brought about by nonspecific hydrophobic interactions*" (Kuwajima, 1989). Previous estimates of the ΔC_p for the molten globule have been somewhat higher (~850 cal/K·mol rather than 326 cal/K·mol) (Pfeil et al., 1986). However those experiments were performed for the acidic molten globule at pH 2. Whether this is a real difference between the two forms of the molten globule awaits further experimentation.

The results presented in this paper for bovine apo-α-lactalbumin are also in agreement with results obtained for the molten globule state of other proteins. For example, Chen et al. (1989) concluded that the ΔC_p between the molten globule transition state and the native state of T4 lysozyme is ~500 cal $(K \cdot mol)^{-1}$ compared to the ΔC_p of ~2200 cal $(K \cdot mol)^{-1}$ between the unfolded and native states. These values are close to the ones in Table I. Recently, Mitaku et al. (1991), using fluorescence spectroscopy, also concluded that the molten globule state of bovine carbonic anhydrase B is stabilized primarily by hydrophobic interactions.

The absence of a significant number of tertiary structure hydrogen bonds coupled to the presence of significant hydrophobic interactions is consistent with the known structural features of the molten globule state. Since structures stabilized by the apposition of purely hydrophobic surfaces lack the rigid geometric constraints created by hydrogen bonds at specific locations in the molecule, they have a larger conformational entropy. It is conceivable that this gain in conformational entropy compensates the loss of tertiary structure hydrogen bonds, resulting in the stabilization of the molten globule state. This larger entropy is also consistent with the recent observation that the molten globule is consistent with a variety of disulfide bond pairings (Ewbank & Creighton, 1991).

ACKNOWLEDGMENTS

We thank Kenneth Murphy for many helpful discussions and Martin Straume for his help with the error analysis. We

also thank Peter Privalov and George Makhatadze for providing us with their GuHCl binding data prior to publication.

References

Baldwin, R. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8069–8072.

Baum, J., Dobson, C. M., Evans, P. A., & Hanley, C. (1989) *Biochemistry 28*, 7–13.

Chen, B., Baase, W. A., & Schellman, J. A. (1989) *Biochemistry 28*, 691–699.

Christensen, H., & Pain, R. H. (1991) *Eur. Biophys. J. 19*, 221–229.

Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotonov, G. V., Venyaminov, S. Y., & Ptitsyn, O. B. (1981) *FEBS Lett. 136*, 311–315.

Dolgikh, D. A., Abaturov, L. V., Bolotina, I. A., Brazhnikov, E. V., Bychkova, V. E., Bushuev, V. N., Gilmanshin, R. I., Levedev, Y. O., Semisotnov, G. V., Tiktopulo, E. I., & Ptitsyn, O. B. (1985) *Eur. Biophys. J. 13*, 109–121.

Ewbank, J. J., & Creighton, T. (1991) *Nature 350*, 518–520.

Freire, E. (1989) *Comments Mol. Cell. Biophys. 6*, 123–140.

Freire, E., & Biltonen, R. L. (1978) *Biopolymers 17*, 463–479.

Hughson, F. M., Wright, P. E., & Baldwin, R. L. (1990) *Science 249*, 1544–1548.

Ikeguchi, M., Kuwajima, K., & Sugai, S. (1986) *J. Biochem. (Tokyo) 99*, 1191–1201.

Jeng, M.-F., Englander, S. W., Elove, G. A., Wand, A. J., & Roder, H. (1990) *Biochemistry 29*, 10433–10437.

Johnson, M. L. (1983) *Biophys. J. 44*, 101–106.

Johnson, M. L., & Frasier, S. G. (1985) *Methods Enzymol. 117*, 301–342.

Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51*, 459–489.

Kim, P. S., & Baldwin, R. L. (1990) *Annu. Rev. Biochem. 59*, 631–660.

Kronman, M. J., & Andreotti, R. E. (1964) *Biochemistry 3*, 1145–1151.

Kuwajima, K. (1977) *J. Mol. Biol. 114*, 241–258.

Kuwajima, K. (1989) *Proteins: Struct., Funct., Genet. 6*, 87–103.

Kuwajima, K., Nitta, K., Yoneyama, M., & Sugai, S. (1976) *J. Mol. Biol. 106*, 359–373.

Kuwajima, K., Ogawa, Y., & Sugai, S. (1981) *J. Biochem. (Tokyo) 89*, 759–770.

Kuwajima, K., Mitani, M., & Sugai, S. (1989) *J. Mol. Biol. 206*, 547–561.

Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431–441.

Mitaku, S., Ishido, S., Hirano, Y., Itoh, H., Kataoka, R., & Saito, N. (1991) *Biophys. Chem. 40*, 217–222.

Murphy. K. P., & Gill, S. J. (1990) *Thermochim. Acta 172*, 11–21.

Murphy, K. P., & Gill, S. J. (1991) *J. Mol. Biol.* (in press).

Nelder, J. A., & Mead, R. (1965) *Comput. J. 7*, 308–313.

Ohgushi, M., & Wada, A. (1983) *FEBS Lett. 164*, 21–24.

Okuda, T., & Sugai, S. (1977) *J. Biochem. (Tokyo) 81*, 1051–1056.

Pace, C. N. (1986) *Methods Enzymol. 131*, 266–280.

Pfeil, W., & Privalov, P. L. (1975) *Biophys. Chem. 3*, 23–32.

Pfeil, W., & Sadowski, M. L. (1985) *Stud. Biophys. 109*, 163–170.

Privalov, P. L., & Gill, S. J. (1988) *Adv. Protein Chem. 39*, 191–239.

Privalov, P. L., & Makhatadze, G. (1990) *J. Mol. Biol. 213*, 385–391.

Ptitsyn, O. B. (1987) *J. Protein Chem. 6*, 272–293.

Ramsay, G., & Freire, E. (1990) *Biochemistry 29*, 8677–8683.

Semisotnov, G. V., Rodionova, N. A., Kutyshenko, V. P., Ebert, B., Blanck, J., & Ptitsyn, O. B. (1987) *FEBS Lett. 224*, 9–13.