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Abstract: The thermal denaturation of α-lactalbumin was studied at pH 7.0 and 9.0 in aqueous 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) by high-sensitivity differential scanning calorimetry. The conformation of the protein was analyzed by a combination of fluorescence and circular dichroism measurements. The most obvious effect of HFIP was lowering of the transition temperature with an increase in the concentration of the alcohol up to 0.30 M, beyond which no calorimetric transition was observed. Up to 0.30 M HFIP the calorimetric and van’t Hoff enthalpy remained the same, indicating the validity of the two-state approximation for the thermal unfolding of α-lactalbumin. The quantitative thermodynamic parameters accompanying the thermal transitions have been evaluated. Spectroscopic observations confirm that α-lactalbumin is in the molten globule state in the presence of 0.50 M HFIP at pH 7.0 and 0.75 M HFIP at pH 9.0. The results also demonstrate that α-lactalbumin in the molten globule state undergoes a noncooperative thermal transition to the denatured state. It is observed that two of four tryptophans are exposed to the solvent in the HFIP induced molten globule state of α-lactalbumin compared to four in the 8.5 M urea induced denatured state of the protein. It is also observed that the HFIP induced molten globule states at the two pH values are different from the acid induced molten globule state (A state) of α-lactalbumin. © 2004 Wiley Periodicals, Inc. Biopolymers 73: 405–420, 2004

Keywords: bovine α-lactalbumin 1,1,1,3,3,3-hexafluoroisopropanol; differential scanning calorimetry; circular dichroism; fluorescence; molten globule

INTRODUCTION

Ever since Levinthal proposed that protein folding cannot be a random process there has been considerable interest in the exact nature of protein folding. Different models such as the hydrophobic collapse model, the framework model, and the funnel model have been proposed. The common feature of these models is the prediction of the presence of an intermediate, although they vary in their structural features in the respective models. Different experiments in recent years have proved that the hypothesis of the
intermediate state is indeed true. Characterization of these intermediates is essential for elucidating the problem of protein folding. The biological importance of these nonnative protein conformations in different stages of denatured conditions and aggregated states cannot be ignored either. The progressively destabilized forms may provide a representation of the folding path if it is assumed that the most stable parts are formed first. The transient nature of these intermediates makes it difficult to isolate and study them. Stopped flow kinetics, protein dissection and mutation, and studies on model peptides are some of the methods used to trap these partially folded intermediates. Nonaqueous solvents are widely used to generate these states in vitro. Alcohols, mild denaturation conditions, and salt solutions have been used for this purpose. Alcohols weaken nonlocal hydrophobic interactions and enhance local polar interactions such as hydrogen bonds in proteins. Trifluoroethanol (TFE) is perhaps the alcohol most used for producing these partially folded states. The presence of the three fluorine atoms makes TFE capable of affecting the interactions in protein. Because the F atoms are instrumental in bringing about the changes in the functionality of ethanol, the interest in fluorinated compounds is rising. In recent years, use of 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) has been reported in the generation of these intermediates and several other applications in biochemical studies. It has a pK$_a$ of 9.3; hence, it is more acidic than its hydrocarbon analogue isopropanol (pK$_a$ = 17.2) and TFE (pK$_a$ = 12.4). The presence of two CF$_3$ groups alters its properties to a great extent. It is a better H-bond donor and poorer H-bond acceptor than TFE. Hence, HFIP is potentially more powerful than TFE in terms of perturbing the H-bonding, and hydrophobic interactions in proteins. HFIP has been used to unfold aggregates of the Alzheimer’s amyloid peptide or prion protein peptides. It has been shown to induce refolding in the case of Coba neurotoxin in the presence of SDS. HFIP has effectively formed aggregates in cecropin AD and molten globule (MG) states in myoglobin. Molten globule has been reported to be actual intermediates in the folding pathway of α-lactalbumin and cytochrome c. These states have a native-like secondary structure (hence globule) but lack the specific, extensive packing of the tertiary structure (hence molten). Bovine α-lactalbumin is a Ca$^{2+}$ bound protein with 123 amino residues, a pI of 4.6, and a molecular weight of 14.2 kDa. It has a well-established crystal structure and its folded conformation in the native state is well known. The molten globule of α-lactalbumin has become the reference point for the studying of stable partially folded proteins. Although HFIP is being used as a structure modifier, systematic study on thermal unfolding of proteins in presence of HFIP in combination with its effect on the conformations of the protein is missing in the literature. To the best of our knowledge there are no quantitative data available on the interaction of HFIP with α-lactalbumin. In this paper we report a combination of calorimetric, fluorescence, and circular dichroism spectroscopy to understand the thermal denaturation of α-lactalbumin in the presence of HFIP quantitatively and show for the first time that HFIP induces a molten globule state that is different from the acid induced A state of the protein.

MATERIALS AND METHODS

Materials

Bovine α-lactalbumin (type I), 1,1,1,3,3,3-hexafluoroisopropanol (>99.5%), Tris (hydroxymethyl) aminomethane (Trizma base, 99.9%), 8-anilino-1-naphthalenesulfonic acid (ANS, >99%), sodium acetate, and glycine (> 99%) of the best available purity grade (mentioned in parentheses) were purchased from Sigma Chemical Company and used without further purification. The acids and bases (HCl and NaOH) were of reagent grade and of more than 99% purity. The water used for preparing the solutions was double distilled followed by deionization using a Cole-Parmer research mixed-bed ion exchange column. The stock solutions of α-lactalbumin for all the experiments were prepared by extensive dialysis of the protein at 4°C against the desired buffer with at least three changes. The reported pH is that of the dialysate, determined using a standard Control Dynamics pH meter at room temperature. The buffers used were 20 mM Tris–HCl and glycine–NaOH at pH 7.0 and 9.0, respectively.

Calorimetry

The thermal denaturation experiments were carried out on a micro DSC from Setaram (France). Here the protein concentration was kept at 352.0 μM and the scan rate used for all the experiments was 30 K h$^{-1}$. Experimental details and conversion of the raw data to excess heat capacity versus temperature plots have been reported earlier. The excess heat capacity versus temperature plots were analyzed by the EXAM program of Kirchhoff and the thermodynamic parameters accompanying the thermal transitions were evaluated. The reversibility of the scans was checked by heating.

1Abbreviations used: HFIP, 1,1,1,3,3,3-hexafluoroisopropanol; TFE, 2,2,2-trifluoroethanol; MG, molten globule; SDS, sodium dodecyl sulfate; ANS, 8-anilino-1-naphthalenesulfonic acid; CD, circular dichroism.
the sample to just above transition temperature, cooling immediately, and then reheating. The thermal denaturation of α-lactalbumin in the presence of varying concentrations of HFIP was performed at pH 7.0 and 9.0.

**UV-Visible Experiments**

The concentration of α-lactalbumin was determined using an extinction coefficient of $E_{280} = 20.1$ in pH 7.0 buffer.\(^{41}\) For the concentration determination and thermal denaturation scans a Jasco-V570 spectrophotometer was used. The protein concentration for the thermal denaturation experiments was kept at 7.0 M and the absorbance at different temperatures was measured at a fixed wavelength of 295 nm.\(^{42}\) The temperature around the cuvettes was controlled with a Jasco ETC-505 T temperature controller unit to within ±0.1 K. To check for aggregation of the protein in solution, the absorbance was checked at different concentrations of the alcohol at 340 nm.

**Fluorescence Experiments**

The fluorescence experiments were done on a Perkin–Elmer LS-55 spectrofluorimeter at 25°C with a quartz cell of 1-cm path length. The protein concentration in all the experiments was kept between 3.5 and 7.0 μM. The excitation and emission slit widths were fixed at 5 nm. The excitation wavelength was kept at 295 nm to selectively excite the tryptophan molecules. For the ANS binding and energy transfer experiments, the ANS concentration was kept at 12 × 10⁻⁵ M using the extinction coefficient of ANS\(^{43}\) as $E_{350} = 5000$ m⁻¹ cm⁻¹ and the protein concentration was 21.0 μM. For ANS binding experiments and energy transfer experiments the excitation wavelength was fixed at 365 and 295 nm, respectively. Quenching experiments were done with acrylamide and KI; the concentration of the quencher was varied from 0.05 to 0.30 M. The time of addition of compounds and reading the fluorescence was typically 30 min. The background spectrum containing the same amount of additive as the sample was subtracted from all plots.

**Circular Dichroism Experiments**

The CD experiments were performed on a Jasco-810 CD spectropolarimeter at 25°C. The protein concentration and path length of the cell used were 10 μM and 0.1 cm for far UV CD and 30 μM and 1 cm for near UV CD. The spectropolarimeter was purged with N₂ prior to the experiment. Each CD plot was an average of three accumulated plots. The plots were baseline corrected. The molar ellipticity was calculated from the observed ellipticity, θ, as \(100 \theta c/l\) where \(c\) is the concentration of the protein solution in molarity and \(l\) is the pathlength of the cell in centimeters.

**RESULTS**

**Thermal Denaturation of α-Lactalbumin in the Presence of HFIP at pH 7.0**

The representative differential scanning calorimetric profiles of thermal denaturation of α-lactalbumin in the absence and presence of varying concentrations of HFIP at pH 7.0 are shown in Figure 1, and the corresponding thermodynamic parameters accompanying the transitions are reported in Table I. In Table I $T_{1/2}$ is the transition temperature where the area
under the transition curve is half complete. \( \Delta H_{\text{cal}}, \Delta C_p, \) and \( \Delta S \) are the calorimetric enthalpy, heat capacity, and entropy of denaturation, respectively, and \( \beta \) is the ratio of van’t Hoff to calorimetric enthalpy. The van’t Hoff enthalpy was determined by the expression

\[
\Delta H(T) = \Delta H_{\text{cal}}(T_{1/2}) + \Delta C_p(T - T_{1/2}) \tag{2}
\]

\[
\Delta G^0 = \Delta H_{\text{cal}}(T_{1/2})[1 - (T/T_{1/2})] + \Delta C_p(T - T_{1/2}) - T\Delta C_p\ln(T/T_{1/2}) \tag{3}
\]

\[
\Delta S^0 = \Delta H_{\text{cal}}/T_{1/2} + \Delta C_p\ln(T/T_{1/2}) \tag{4}
\]

By these interpolations in temperature, thermodynamic parameters are obtained as a function of alcohol concentration at constant temperatures (303.15, 313.15, and 323.15 K). The values follow smooth curves with a minima in \( \Delta S^0 \) and \( \Delta H \) (Figure 2.). The calorimetric data obtained from the reversible thermal denaturation were used to calculate the denaturational change in the preferential solvation parameter of component 2 by component 3 (\( \Delta \Gamma_{23} \)) as shown in Table II using the equation

\[
\Delta \Gamma_{23} = \Gamma_{D3} - \Gamma_{N3} = -\frac{\Delta H\left(\frac{\partial T_{1/2}}{\partial x_3}\right)_{\text{ph}}}{RT_{1/2}\left(\frac{\alpha \ln \chi}{\partial \chi}\right)_{T_{1/2}}} \tag{5}
\]

Where, \( \Gamma_{D3} \) and \( \Gamma_{N3} \) are the preferential solvation of the protein (component 2) by HFIP (component 3) in the denatured and native states, respectively. Here \( x \) is the mole fraction and \( a \) is the activity of alcohol. The highest mole fraction of alcohol used in the calorimetric experiments is 0.0055; therefore, the activity was taken to be equal to the concentration. The positive sign of \( \Delta \Gamma_{23} \) indicates that HFIP favors the denatured protein conformation relative to the native state studied up to 0.30 M.

### Circular Dichroism

Since no thermal transitions for \( \alpha \)-lactalbumin were observed in the presence of HFIP above 0.30 M of
CD experiments were carried out at pH 7.0 in all the concentrations of HFIP used for the DSC and also at higher concentrations. The far and near UV CD plots for different concentrations of HFIP at pH 7.0 are shown in Figure 3. From Figure 3 (a) it is seen that α-lactalbumin has a negative...
band near 208 nm and a relatively flat region of negative ellipticity between 210 and 225 nm, which is in agreement with that reported in the literature.\(^{45}\) In the presence of HFIP the \(\alpha\)-helix is found to be increasing in strength as is seen from the increased negative intensity of the band near 208 nm till 0.50\(M\) HFIP and beyond that there is a shift of the major bands. The positive band at 192 nm also shifts to 198 nm and a sharp minima at 230 nm is observed. The near UV CD of the protein, shown in Figure 3(b), is the characteristic of \(\alpha\)-lactalbumin.

Uncertainties reported are twice the uncertainties determined by including a 2\% error in calorimetric enthalpies and 0.1 K in transition temperature.

![Figure 3](image)

**FIGURE 3**  (a) Far-UV CD spectra of 30\(\mu\)M \(\alpha\)-lactalbumin at pH 7.0 and 25\(^\circ\)C in buffer (A) and in the presence of varying concentrations of HFIP: 0.10\(M\) (B), 0.25\(M\) (C), 0.50\(M\) (D), 0.75\(M\) (E), 1.0\(M\) (F), and 2.5\(M\) (G). (b) Near UV CD spectra of 30\(\mu\)M \(\alpha\)-lactalbumin at pH 7.0 and 25\(^\circ\)C in buffer (A) and in the presence of varying concentrations of HFIP: 0.10\(M\) (B), 0.25\(M\) (C), 0.50\(M\) (D), 0.75\(M\) (E), 1.0\(M\) (F).

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**Table II**  Denaturational Change in the Preferential Solvation Parameter (\(\Delta\Gamma_{32}\)) at the Thermal Unfolding of \(\alpha\)-Lactalbumin at pH 7.0

<table>
<thead>
<tr>
<th>Mole fraction of HFIP</th>
<th>(\Delta\Gamma_{32})</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0009</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>0.0018</td>
<td>2.8 ± 0.1</td>
</tr>
<tr>
<td>0.0037</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>0.0055</td>
<td>7.6 ± 0.3</td>
</tr>
</tbody>
</table>
with a trough near 252 nm and negative maxima at 272 nm. This spectrum is consistent with the presence of optically active cystine and aromatic chromophores. After addition of increasing amounts of HFIP, the negative ellipticity at 272 nm is progressively decreased. The change in the band height of the CD plot indicates that tryptophan residues, which contribute to this band, have an increased freedom of movement. The change in the band near 252 nm can be ascribed to the conformational changes occurring in the vicinity of the disulfide bond as a result of the conformational changes in the protein. At 0.50 M HFIP and beyond the tertiary structure is totally disrupted (Figure 3). The presence of 2.50 M HFIP in \( \alpha \)-lactalbumin resulted in a turbid solution.

Fluorescence

To check the difference in conformations, intrinsic fluorescence of the protein under conditions similar to those above was studied. Figure 4 gives the intrinsic fluorescence of \( \alpha \)-lactalbumin in different concentrations of HFIP at pH 7.0. In the absence of HFIP the fluorescence spectra of the protein gives the characteristic \( \lambda_{\text{max}} \) of \( \alpha \)-lactalbumin at 331 nm, which is consistent with that obtained in the literature.\(^{46}\) In the presence of 0.25 M HFIP there is a shift of the \( \lambda_{\text{max}} \) from 331 to 344 nm. At 0.50 M HFIP there is a further red shift to 351 nm. Beyond that there is a slight blue shift to 348 nm along with a decrease in the fluorescence intensity. For the 8.5 M urea denatured protein, \( \lambda_{\text{max}} \) at 354 nm corresponding to a fully denatured protein was obtained. To further investigate the HFIP induced conformations acrylamide quenching experiments were performed. For dynamic or collisional quenching the process is governed by the equation\(^{47}\)

\[
\frac{F_0}{F} = 1 + K_{SV}[Q],
\]

where \( F_0 \) is the fluorescence intensity in absence of a quencher, \( F \) is the intensity in the presence of the quencher at concentration \([Q]\), and \( K_{SV} \) is the Stern–Volmer quenching constant. The plot is shown in Figure 5 and the \( K_{SV} \) values are obtained.
from the slope. In the presence of 0.50 M HFIP, the $K_{SV}$ value (5.93 M$^{-1}$) is between that for the native (2.13 M$^{-1}$) and denatured forms (7.24 M$^{-1}$) of the protein. The quenching experiments were also done in presence of KI. It was found that in the native state KI was not able to quench the fluorescence of lactalbumin but in the presence of 0.50 M HFIP it showed a considerable amount of quenching. When $(F_0/F - 1)$ was plotted against $[Q]$ an upward curving plot was obtained in the presence of 0.50 M HFIP; hence, to interpret the result the modified Stern–Volmer equation$^{48}$ was used:

$$F_0/(F_0 - F) = 1/f_a + 1/(f_a \cdot K_a \cdot [Q]),$$

(7)
where $f_a$ is the fraction of fluorophore accessible to the quencher, and $K_a$ is the association constant of the quencher to the fluorophore. When $[F_0/(F_0 - F)]$ was plotted against $1/[Q]$ a straight line was obtained (Figure 5). The intercept gives the reciprocal of $f_a$, which is the fraction of tryptophans accessible to the quencher and its $(f_a^{-1})$ value was 0.49. In the presence of 8.5 M urea the plot of $(F_0/F - 1)$ was again found to be linear. ANS binding and energy transfer studies were done to monitor the conformational changes in the protein (Figure 6). It was observed that the ANS intensity gradually increases on addition of HFIP, being maximum in presence of 0.50 M HFIP. The $\lambda_{\text{max}}$ of the ANS intensity also shifts from 510 to 480 nm.

**UV-Visible**

To investigate the sudden shift in the CD plots beyond 0.75 M HFIP (Figure 3) and decrease in the fluorescence intensity (Figure 4) the UV absorbance at 340 nm was used to check for aggregation. The plot (Figure 7) shows that beyond 1.0 M HFIP there is a sharp increase in the absorbance at 340 nm. This is a characteristic of protein aggregation. Hence, aggrega-
Thermal Denaturation of $\alpha$-Lactalbumin in the Presence of HFIP at pH 9.0.

The results on the thermal denaturation of $\alpha$-lactalbumin at pH 9.0 are given in the electronic supporting information. The results show a trend similar to that observed at pH 7.0. The MG state in this case was obtained in the presence of 0.75 M HFIP as is seen in the far and near UV CD spectra of $\alpha$-lactalbumin in the presence of HFIP at pH 9.0 (Figure 8). The relative change in the transition temperature of the protein upon addition of HFIP at pH 7.0 and 9.0 is compared in Figure 9.

Comparative Study of Different MG States

In order to compare the MG states obtained by us with the acid induced MG state of $\alpha$-lactalbumin (A state) we calculated the percentage of $\alpha$-helicity ($f_h$) by the following method,$^{46}$ involving, $\theta_C$ (molar ellipticity for pure coil), $\theta_{411}$ (molar ellipticity for pure helix), and $\theta_{222}$ (the molar ellipticity at the wavelength 222 nm):

$$f_h = \frac{\theta_{222} - \theta_C}{\theta_{411} - \theta_C},$$

where $\theta_C = 2220 \times (377)$ and $\theta_{411} = (-44,000 + 250 T)$ (1–3/N). $T$ is temperature in degrees Celsius, and $N$ is the number of amino acid residues in the concerned protein. The results are given in Table III. The far and near UV CD spectra of the acid induced $\alpha$-lactalbumin and the $\alpha$-helicity were calculated from the experimentally obtained spectra in 20 mM glycine–HCl buffer. The percentage of $\alpha$-helicity is similar to that reported.$^{46}$ The results show that the alcohol induced MG states have a similar degree of $\alpha$-helicity, which is different from that of the A state. The CD spectra of the overlapping plots of the HFIP induced MG states and the acid induced A state are given in Figure 10. It is seen that the secondary structure content in the A state is less than that of the alcohol induced state and the difference in the tertiary structure is also significant. The molar ellipticity value at 272 nm is much higher in the acid induced state compared to the alcohol induced states.

DISCUSSION

The reversibility and two-state character of the thermal unfolding of $\alpha$-lactalbumin in aqueous HFIP up to 0.30 M of the latter have been demonstrated by the data presented in Table I ($\beta = 0.98 \pm 0.04$). Therefore, it is permissible to apply equilibrium thermodynamics for the evaluation of thermodynamic parameters as a function of temperature and alcohol concentration. Here we have neglected any possible effects on the charged state of the protein due to lowering of the dielectric constant by the addition of alcohols to the aqueous solvent. The most obvious effect of HFIP is lowering of the thermal transition temperature of
the protein up to 0.30 M HFIP, beyond which no transition is detected.

Large and positive values of $\Delta C_p$ for protein denaturation are largely due to the exposure of the nonpolar groups that are previously buried in the native structure to the surrounding solvent, hence leading to the ordering of the solvent molecules around them. In the absence of HFIP $\Delta C_p$ for $\alpha$-lactalbumin is 4.6 ± 0.3 kJ K$^{-1}$ mol$^{-1}$, which indicates a significant contribution from the exposure of the apolar groups to the buffer medium. The average value of $\Delta C_p$ in the presence of HFIP is 2.6 ± 0.4 kJ K$^{-1}$ mol$^{-1}$, which indicates that upon unfolding of the $\alpha$-lactalbumin globule in the aqueous HFIP solution, the solvation of the nonpolar side chain groups and hence reordering of water structure around the nonpolar residues is less compared to that in the buffer. This is due to the interaction of the exposed hydrophobic groups of the protein upon denaturation with the $\text{–CF}_3$ group of HFIP.

**FIGURE 8** (a). Far UV CD spectra of 30 $\mu$M $\alpha$-lactalbumin at pH 9.0 and 25°C in buffer (A), and in the presence of varying concentrations of HFIP: 0.10 M (B), 0.25 M (C), 0.50 M (D), 0.75 M (E), 1.0 M (F), and 2.5 M (G). (b) Near UV CD spectra of 30 $\mu$M $\alpha$-lactalbumin at pH 9.0 and 25°C in buffer (A) and in the presence of varying concentrations of HFIP: 0.10 M (B), 0.25 M (C), 0.50 M (D), 0.75 M (E), 1.0 M (F).
molecules. Thermodynamic functions $\Delta H$, $\Delta S^0$, and $\Delta G^0$ were calculated at 303.15, 313.15, and 323.15 K. The values of $\Delta G^0$ show a uniform decrease with increasing concentration of alcohol. The values of both $\Delta H$ and $\Delta S^0$ pass through a minima (Figure 2). The minima in both the cases at all the temperatures studied is at 0.10 M HFIP. These trends indicate the stabilization of the denatured state relative to the native state with increasing concentrations of HFIP in solution. Also, as expected the value of $\Delta G^0$ decreases with increasing temperature.

When the native protein denatures, its surface changes its character; hence, the preferential solvation of the macromolecule by the surrounding solvent also changes. Denaturational change in the preferential solvation parameter ($\Delta \Gamma_{23}$) was found to be positive (Table II). The $\Delta \Gamma_{23}$ (mole/mole basis) value also increases with increasing HFIP concentration, suggesting that in the denatured state more HFIP molecules are situated near the protein compared to the native state. This indicates that HFIP has greater affinity to the protein surface exposed upon denaturation than that of water and thus will shift the native (N)$\rightleftharpoons$ denatured (D) equilibrium to the right. This is in agreement with the microcalorimetric evidence that HFIP decreases the thermal stability of lactalbumin. As mentioned earlier, no thermal transition was observed in $\alpha$-lactalbumin in the presence of HFIP at concentrations higher than 0.30 M. The enhanced secondary structure of $\alpha$-lactalbumin at pH 7.0 in the presence of 0.50 M HFIP and loss of tertiary structure indicate that $\alpha$-lactalbumin is in the MG state under these conditions (Figure 3). There have been contradictory reports available in the literature on the cooperativity in the thermal unfolding of $\alpha$-lactalbumin in the molten globule state.50,51 Our results indicate that the molten globule state of $\alpha$-lactalbumin undergoes noncooperative thermal unfolding to the denatured state as no thermal transition was observed in the presence of 0.50 M HFIP.

Characteristically, MG states have a $\lambda_{max}$ in between the $\lambda_{max}$ of the fully native and fully denatured state.46 Our experimental results also show that at pH 7.0, from 0.25 M HFIP onward the $\lambda_{max}$ is between 331 nm(corresponding to the fully native protein) and 354 nm (corresponding to the fully denatured protein). But from the corresponding CD spectra it is seen that apart from the conformation present in 0.50 M HFIP none of them satisfies the criteria of a MG state, so the rest of the conformations can be termed “intermediate states.”

![Figure 9](image-url) Plot of $\Delta T_{1/2}$ ($T_{1/2}$ in the presence of cosolvent - $T_{1/2}$ in presence of buffer) against the concentration of HFIP at pH 7.0 (■) and pH 9.0 (●).

<table>
<thead>
<tr>
<th>pH</th>
<th>Concentration of HFIP</th>
<th>Percentage of $\alpha$-helicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>0.00</td>
<td>$30.5 \pm 0.6$</td>
</tr>
<tr>
<td>7.0</td>
<td>0.50</td>
<td>$44.9 \pm 0.9$</td>
</tr>
<tr>
<td>9.0</td>
<td>0.75</td>
<td>$48.1 \pm 1.0$</td>
</tr>
</tbody>
</table>
Formation of the MG state is also supported by the quenching of fluorescence. The extent of quenching and hence the $K_{SV}$ value depends on the degree to which the quencher achieves the encounter distance of the fluorophore. The linear plot of $F_0/F$ against $[Q]$ (Figure 5) indicates that all the four tryptophan residues of the protein are equally accessible to the quencher. This is possible because acrylamide can penetrate both the hydrophobic and the hydrophilic region. The Stern–Volmer quenching constant for the native protein was found to be $2.13 M^{-1}$, which is similar to the values of this constant found in many proteins in the native state. For the denatured protein the value is $7.24 M^{-1}$, almost 3.4 times higher than that of the native protein. The values suggest that in the native state the tryptophans are shielded from the quencher to the highest degree and in the denatured state they are shielded the least. In the presence of $0.5 M$ HFIP the quenching constant is between that of the native and denatured states ($5.93 M^{-1}$), suggesting an intermediate conformation.

**FIGURE 10** (a) Far UV CD spectra of the A state of lactalbumin (A) and the MG state obtained in the presence of $0.50 M$ HFIP at pH 7.0 (B) and in the presence of $0.75 M$ HFIP at pH 9.0 (C). The temperature was 25°C. (b) Near UV CD spectra of the A state of lactalbumin (A) and the MG state obtained in the presence of $0.50 M$ HFIP at pH 7.0 (B) and in the presence of $0.75 M$ HFIP at pH 9.0 (C). The temperature was 25°C.
The quenching experiments were also done in the presence of KI. KI, as an ionic compound, can encounter and hence quench only those tryptophans which are exposed to the solvent. α-Lactalbumin has four tryptophan residues at positions 26, 60, 104, and 118. It has been reported that of these four tryptophans, the one at position 60 is part of a loop and is exposed to the solvent in the native state. It contributes only 7% to the total fluorescence of the protein; hence, in the native state the quenching of the protein fluorescence by KI is almost nil. The tryptophan residues at positions 104 and 118 are a part of the \( \beta \)-lactalbumin helix and 26 is a part of the \( \alpha \)-helix. These three residues are essential for the formation of the MG-like structure. The upward curving plot of \( (F_0/F - 1) \) against [KI] in the presence of 0.50M HFIP confirms that KI is experiencing fluorophores in a different environment. Applying the modified Stern–Volmer equation it is shown that of the four tryptophans 49%, i.e., two, are exposed to the solvent in the HFIP induced MG state. It has been shown by hydrogen exchange of tryptophan indole protons, paramagnetic perturbation of the NMR spectra, that tryptophans in positions 26 and 104 are buried in the native state as well as in the acid induced MG state. In the presence of 8.5M urea the linearity of \( (F_0/F - 1) \) against the [KI] plot confirms the fact that under denaturing conditions all four tryptophan residues are subjected to a similar degree of fluorescence quenching.

ANS binding and energy transfer experiments also proved that in the presence of 0.50M HFIP α-lactalbumin is in the molten globule state. When ANS binds to the exposed hydrophobic residues in the protein there is energy transfer between the tryptophans of the protein and the bound ANS. The tryptophan residues lose their fluorescence intensity and the ANS fluorescence gains intensity. From the plots for ANS binding studies (Figure 6a) it is observed that in the native state of the protein, binding is almost nil, gradually increases upon addition of HFIP, and is maximum in the presence of 0.50M HFIP. There is a shift of the \( \lambda_{\text{max}} \) of ANS fluorescence from 510 nm, corresponding to free ANS to 470 nm corresponding to protein bound ANS. From the plots of energy transfer (Figure 6b) it is seen that the intensity of ANS is almost nil in the native state of α-lactalbumin. In the presence of HFIP the fluorescence intensity of ANS increases and that of the protein decreases, indicating that as the protein unfolds ANS binds to the hydrophobic residues of the proteins, facilitating the energy transfer from tryptophans to bound ANS. Here also the intensity of ANS is highest in the presence of 0.50M HFIP. These observations are consistent with the well-established fact that ANS binds more to the MG state compared to the native and denatured states.

From Figure 7 it is clear that α-lactalbumin starts aggregating in presence of 1.0M HFIP, which increases at 2.5M HFIP. As the concentration of alcohol increases in aqueous solution, they associate so as to minimize their contact with water, leading to the formation of micelle-like assemblies with hydrophobic groups inside, but without any macroscopic phase separation. Direct interaction with these micellar structures has been proposed by Melittin to be a reason for the formation of a helix. Still higher concentrations of HFIP can lead to precipitation of the protein by strong interaction between these hydrophobic aggregates and the hydrophobic part of the proteins.

The experiments at pH 9.0 showed results that were similar to that at pH 7.0. The DSC data in Table I of the supporting information showed that till 0.30M thermal denaturation of the protein follows the two-state reversible model. Beyond that it didn’t show any transition. From the CD spectra (Figure 8) it is seen that the native state at pH 9.0 resembles that at pH 7.0 and the \( \alpha \)-helicity increases with increasing concentration of HFIP. It is highest in the presence of 0.75M HFIP. The tertiary structure is totally destroyed at 0.50M HFIP and beyond. The data supported the existence of the molten globule state of the protein in the presence of 0.75M HFIP. This fact was proved by ANS binding and energy transfer experiments, where the highest intensities were obtained in the presence of 0.75M HFIP. Acrylamide quenching experiments gave an intermediate value of the Stern–Volmer quenching constant in the presence of 0.75M HFIP. The tables and figures demonstrating the reaction at pH 9.0 are included in the supporting information.

α-Lactalbumin at pH 9.0 is in a less stable conformation than at pH 7.0 because the transition temperature of the protein in the native state is more at pH 7.0 than at pH 9.0. Hence, it is expected to be more susceptible to the effect of HFIP at pH 9.0 than at pH 7.0. But as seen in Figure 9, that is not so. The relative fall in transition temperature is more in the case of pH 7.0. The MG state, with a similar amount of \( \alpha \)-helicity (Table III), is obtained in the presence of 0.75M HFIP at pH 9.0 compared to 0.50M at pH 7.0. The same trend is observed in the value of \( \Delta T_{23} \) at both pH values. The excess number of HFIP molecules in the denatured state is more at pH 7.0 than at pH 9.0. These observations indicate that HFIP is able to interact more strongly with α-lactalbumin at neutral pH compared to alkaline pH, thus supporting the idea that charge effect is important in the HFIP–α-lactalbumin interaction.
When the far and near UV CD plots for HFIP induced MG states at pH 7.0 and 9.0 and the acid induced A state were overlapped, we saw that the secondary and tertiary structures are almost the same for the alcohol induced states but are different from the A state (Figure 10). It was observed that the percentage of α-helicity for the experimentally obtained MG states is similar but the A state has a lower α-helicity content. The higher molar ellipticity value in the A state at 272 nm suggests that the tryptophan residues have a greater degree of freedom in the A state than in the HFIP induced states. Hence, it may be concluded that the A state with its lesser α helicity and greater degree of freedom for the tryptophan moieties is different from the HFIP induced MG states. The difference in the A state and HFIP induced molten globule state is due to a different solvent environment around the protein. Understanding the mechanism by which HFIP induces the intermediate state requires further experimental investigation.

**CONCLUSION**

The differential scanning calorimetry on the interaction of HFIP with α-lactalbumin shows that at lower concentrations (up to 0.30 M HFIP) the thermal denaturation of the protein follows a two-state (native ⇔ denatured) reversible process as reflected by the equality of van’t Hoff and calorimetric enthalpies. Quantitative thermodynamic parameters, enthalpy, entropy, and heat capacity accompanying the thermal denaturation of α-lactalbumin in the absence and presence of HFIP have been evaluated. The preferential interaction parameters of HFIP with α-lactalbumin confirm that greater interaction of the hydrophobic groups of the alcohol with that of the protein upon denaturation is responsible for the reduced thermal stability of the protein in the presence of the alcohol. At higher concentrations of HFIP intermediate states were obtained, with different secondary and tertiary structure content compared to the native state of the protein. Among them, the molten globule state, which is an equilibrium intermediate in the protein folding pathway, was also found in the presence of 0.50 M HFIP at pH 7.0 and 0.75 M HFIP at pH 9.0 and was confirmed by fluorescence and circular dichroism results. Two of four tryptophans are exposed to the solvent in the HFIP induced molten globule state of α-lactalbumin. The results suggest that α-lactalbumin in the MG state does not undergo any cooperative thermal transition. The MG state induced by the alcohol was found to be different from that of the acid induced MG state of the protein. Comparison of the results at pH 7.0 and 9.0 also suggests that the charge on the protein affects the α-lactalbumin–HFIP interaction.

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**Supporting Information Available:**

The tables and figures corresponding to the experiments performed on α-lactalbumin in the presence of HFIP at pH 9.0 are given in the supporting information available at http://www.interscience.wiley.com/jpages/0006-3525/suppmat/2004/73/v73.4.405.html

**REFERENCES**