2,2,2-Trifluoroethanol-Induced Molten Globule State of Concanavalin A and Energetics of 8-Anilinonaphthalene Sulfonate Binding: Calorimetric and Spectroscopic Investigation

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The interaction of 2,2,2-trifluoroethanol (TFE) with concanavalin A has been investigated by using a combination of differential scanning calorimetry, isothermal titration calorimetry (ITC), circular dichroism (CD), and fluorescence spectroscopy at pH 2.5 and 5.2. All of the calorimetric transitions at both the pH values were found to be irreversible. In the presence of 4 mol kg^{-1} TFE at pH 2.5, concanavalin A is observed to be in a partially folded state with significant loss of native tertiary structure. The loss of specific side chain interactions in the transition from native to the TFE-induced partially folded state is demonstrated by the loss of cooperative thermal transition and reduction of the CD bands in the aromatic region. Acrylamide quenching, 8-anilinonaphthalene sulfonate (ANS) binding, and energy transfer also suggest that in the presence of 4 mol kg^{-1} TFE at pH 2.5 concanavalin A is in a molten globule state. ITC has been used for the first time to characterize the energetics of ANS binding to the molten globule state. ITC results indicate that the binding of ANS to the molten globule state and acid-induced state at pH 2.5 displays heterogeneity with two classes of non-interacting binding sites. The results provide insights into the role of hydrophobic and electrostatic interactions in the binding of ANS to concanavalin A. The results also demonstrate that ITC can be used to characterize the partially folded states of the protein both qualitatively and quantitatively.

1. Introduction

Due to a large number of possible conformations of a polypeptide chain it is impossible for a protein to find its native state by random search. Therefore it has been proposed that there is a specific folding pathway, in which a protein has to go through a sequence of intermediates to fold into the native structure.[1] To understand fully the conformational behavior of a protein, it is necessary to define not only the structure of its native state but also those of various intermediate states. Thus detection and characterization of protein folding intermediates have been central to protein folding studies.

The study of partially folded conformations induced by extreme solvent conditions, such as low pH or addition of alcohols, is thought to provide a key to understanding the mechanism of protein folding.[2−12] The structures of the observed partially folded states are believed to give information on early events in the folding process. 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 states.[13−17] The molten globule (MG) state is one of the well-studied intermediate states.[18−21] This state differs from the native state by the absence of close packing throughout the molecule and by a substantial increase in fluctuations in the side chains as well as of larger parts of the molecules. Studies have revealed the biological significance of MG states in processes such as chaperon binding[22,23] and transport across membranes.[24]

Nonaqueous solvents are widely used to generate these states in vitro. Alcohols, mild denaturing conditions, and salt solutions have been used extensively for this purpose.[2−6] 2,2,2-Trifluoroethanol is perhaps the alcohol most used for producing these partially folded states.[8−12] The presence of three fluorine atoms makes TFE capable of affecting the interactions in proteins. Little is known about the effect of TFE on β-sheet structures although there is evidence of β-sheet-to-α-helix conversion in some proteins and peptides.[25]

Lectins are important tools in biomedical research; hence it is of interest to study their conformational properties in different solvent environments. Concanavalin A is a lectin isolated from jack beans that exists as a tetramer at physiological pH and as a dimer at pH values less than 6.[26,27] Each monomer (M_r = 26 500) consisting of four tryptophanyl residues possesses one saccharide binding site as well as a transition metal ion site S1 that typically binds M_n^{2+} and a site S2 that binds Ca^{2+}.[28] The three-dimensional structure of the lectin at 1.75 Å resolution has been determined by X-ray diffraction analysis. Our earlier studies[29] have shown that thermal unfolding of concanavalin A at pH 5.2 is irreversible and scan-rate-dependent. In this paper we report a combination of calorimetric, fluorescence, and circular dichroism spectroscopy to explore the thermal unfolding of concanavalin A in the presence of TFE quantitatively and have shown that TFE induces a molten globule state in concanavalin A at pH 2.5. Isothermal titration Calorimetry has been used for the first time to determine the energetics of binding of 8-anilinonaphthalene sulfonate (ANS) to the acid-induced state and molten globule state of concanavalin A

2. Materials and Methods

Concanavalin A, sodium acetate, ANS, glycine, and 2,2,2 trifluoroethanol of the best available purity grade were purchased from Sigma-Aldrich Chemical Co.. Sodium acetate, sodium chloride, manganese chloride, and calcium chloride were extra pure analytical reagent grade and obtained from Merck. The mass fraction purity of TFE checked by gas chromatography

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was 0.99. A Sartorius BP 211D digital balance of readability ±0.01 mg was used for the mass measurements. The water used for preparing the solutions was double-distilled and then deionized using a Cole-Parmer research mixed-bed ion exchange column. The protein was dialyzed extensively against 20×10^{-3} mol dm^{-3} glycine−HCl at pH 2.5 and 20×10^{-3} mol dm^{-3} sodium acetate at pH 5.2 containing 1×10^{-3} mol dm^{-3} calcium chloride and 0.1 mol dm^{-3} manganese chloride with at least four changes of the buffer. The reported pH is that of the dialysate measured on a Standard Control Dynamics pH meter at room temperature. The concentration of concanavalin A was determined spectrophotometrically on a Shimadzu double-beam UV 265 spectrometer at 280 nm using $A^{1\%,1cm} = 12.4$ [30] expressed in terms of the monomer.

2.1. Differential Scanning Calorimetry. The thermal denaturation experiments were performed on a micro-differential scanning calorimeter (SETARAM, France) equipped with removable Hastelloy C-276 fluid tight batch cells of 1 cm^3 in capacity. Before being loaded into the calorimetric cells, all of the solutions were degassed. Any loss in water due to evaporation, determined from the mass of the sample before and after degassing, was compensated for by the addition of an appropriate amount of degassed deionized water. The volume of the sample solution in the cell was fixed at 0.85 cm^3, and the weights of the sample and reference cells containing respective solutions were always matched to within 0.1 mg. The reference solution also contained TFE when the measurements were made in the presence of alcohol. In the differential scanning calorimetry (DSC) measurements at pH 5.2, 2 mol dm^{-3} urea was added to keep the transition temperature of the protein within the measurable range of the instrument. The scan rate used in all the experiments was 30 K h^{-1}. The excess power thermal scans were also corrected for the thermal lag of the calorimeter and then converted to an excess heat capacity versus temperature scan by dividing by the scan rate. The corrected DSC data were analyzed by the EXAM program of Kirchoff.[31] The calorimetric reversibility of the thermal transitions was determined by heating the sample to a temperature that is a little over the transition maximum, cooling immediately, and then reheating.

2.2. Isothermal Titration Calorimetry. Isothermal titration calorimetry (ITC) measurements were carried out on a VP−ITC titration microcalorimetry system (MicroCal, Northampton, MA). All solutions were thoroughly degassed before use by stirring under vaccum in a Thermovac unit supplied with the instrument. Titrations were carried out using a 250 μL syringe filled with the solution of interest. The sample cell contained 0.15×10^{-3} mol dm^{-3} protein in the presence of 4 mol kg^{-1} TFE, and the reference cell was filled with degassed buffer. Sequential titrations were performed to ensure full occupancy of the binding sites by loading and titrating with the same ligand without removing the samples from the cell, until the titration signal was essentially constant. The titrations were linked together for data analysis using ConCat 32 software provided by MicroCal, Inc. Control experiments were done to correct the data for the heats of dilution of the ligand, protein, and buffer mixing. The heat released by dilution was negligible. The binding of ANS to the native and the molten globule states of concanavalin A was analyzed using two classes of independent binding sites. This model defines the equilibrium association constants as

$$K_1 = \theta_1/(1 - \theta_1)L \quad (1)$$

$$K_2 = \theta_2/(1 - \theta_2)L \quad (2)$$

where θ is the fractional ligand occupancy

$$L_t = L + M_1(n_1\theta_1 + n_2\theta_2) \quad (3)$$

L_t is the total ligand concentration, and K is the apparent association constant

$$L_t = L + n_1M_tLK_1/(1 + LM_t) + n_2M_tLK_2/(1 + LK_2) \quad (4)$$

The heat content Q after any ith injection is then expressed as

$$Q_i = M_tV_0(n_1\theta_1\Delta H_1 + n_2\theta_2\Delta H_2) \quad (5)$$

Binding models were fitted to the data by a nonlinear least-squares Marquardt algorithm until constant χ^2 values were achieved.

2.3. Circular Dichroism Experiments. The circular dichroism (CD) experiments were performed on a Jasco-810 CD spectropolarimeter. 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 N_2 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 $(100\theta)/(cl)$ where c is the concentration of the protein solution in mol dm^{-3} and l is the path length of the cell in cm.

2.4. Fluorescence Experiments. The fluorescence experiments were done on a Perkin-Elmer LS-55 spectrofluorimeter. The protein concentration in all the experiments was kept at 0.5×10^{-6} mol dm^{-3}. 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 6×10^{-5} mol dm^{-3} using the extinction coefficient of ANS as $E_{350}^{1\%} = 5000$ M^{-1} cm^{-1}, and the protein concentration was 2×10^{-6} mol dm^{-3}. 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 from 50×10^{-3} to 600×10^{-3} mol dm^{-3}, and background spectra containing the same amount of additive were subtracted from all of the plots.

3. Results and Discussion

3.1. Interaction of TFE with Concanavalin A at pH 5.2. The representative differential scanning calorimetric profiles of thermal denaturation of concanavalin A in the absence and presence of varying molalities of TFE at pH 5.2 are shown in Figure 1, and the corresponding two thermodynamic parameters accompanying the transitions are reported in Table 1.

In this table, $T_{1/2}$ is the temperature where the area under the transition curve is half complete, and ΔH_{cal} represents the calorimetric enthalpy of denaturation. Since all the thermal transitions in the absence and presence of TFE were found to be irreversible, laws of equilibrium thermodynamic analysis cannot be applied to extract different thermodynamic parameters.

It is observed that in the presence of 0.1 mol kg^{-1} TFE there is no change in the transition temperature, but beyond 0.1 mol kg^{-1} TFE there is a gradual decrease in the transition temperature. The enthalpy of denaturation in the presence of TFE up to 2.0 mol kg^{-1} was found to be greater than in the absence of TFE.

CD spectra did not reveal the presence of an intermediate state at pH 5.2. As seen from Figure 2, up to 3 mol kg^{-1} TFE there is an increase in the β-sheet content, beyond which it decreases.

The near-UV CD spectra shown in Figure 3 consist of essentially of two positive bands at 265 and 291 nm. With an

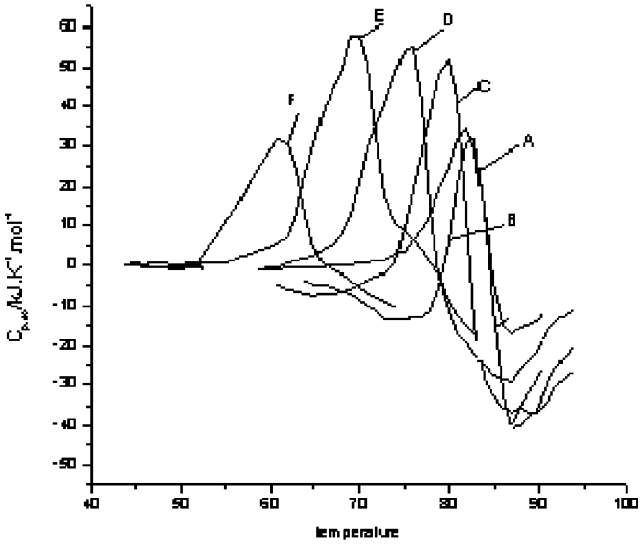

Figure 1. Thermal unfolding of 0.19×10^{-3} mol dm^{-3} concanavalin A in the presence of varying molalities of TFE: (A) buffer, (B) 0.1, (C) 0.5, (D) 1.0, (E) 2, and (F) 5.0 mol kg^{-1}.

TABLE 1: Thermodynamic Parameters Accompanying Thermal Unfolding of 0.19×10^{-3} mol dm^{-3} Concanavalin A at pH 5.2 in the Presence of TFE at a Scan Rate of 0.5 K min^{-1} [a]

TFE (mol kg^{-1})	$T_{1/2}$ (K)	ΔH_{cal} (kJ mol^{-1})
0	355.5	252.5
0.1	355.8	314.0
0.5	352.9	456.0
1.0	349.2	434.3
2.0	342.1	414.0
5.0	332.3	206.9

[a] When errors in sample preparation, reproducibility, and sample impurities are incorporated, the errors in the values of $T_{1/2}$ and ΔH_{cal} are ± 0.1 K and 2%, respectively.

increase in the molality of TFE, there is a concentration-dependent decrease in the tertiary structural content.

Intrinsic fluorescence was done to monitor the change in tryptophan environment in the presence of increasing molalities of TFE. In the presence of TFE, emission spectra did not show changes in intensity, and there was no shift in the wavelength maximum (Figure 4).

3.2. Interaction of TFE with Concanavalin A at pH 2.5. The representative differential scanning calorimetric profiles of thermal denaturation of concanavalin A in absence and presence of varying concentration of TFE at pH 2.5 are shown in Figure 5, and the corresponding two thermodynamic parameters accompanying the transitions are reported in Table 2.

Concanavalin A at pH 2.5 unfolds at 330.8 K. In the presence of increasing molalities of TFE, its transition temperature gradually decreases. Between 3.5 and 4 mol kg^{-1} TFE no endotherm was obtained in the temperature range of 10−70 °C. All of the thermal transitions in the absence and presence of alcohols were found to be irreversible.

CD spectra of concanavalin A in the presence of TFE at pH 2.5 in the near-UV region (Figure 6) show that there is not much change up to 3 mol kg^{-1} TFE. However, above 3 mol kg^{-1} there was a decrease in the near-UV CD signal, and at 4 mol kg^{-1} TFE there was a considerable loss of tertiary structure. Above 4 mol kg^{-1} there was a further reduction in the tertiary structure. Thus, far-UV CD spectra at pH 2.5 (Figure 7) show enhancement in the secondary structural content up to 4.5 mol kg^{-1}. Taken together these results indicate the loss of tertiary structure interactions but substantial retention of secondary structure in the acid-denatured (pH 2.5) state of concanavalin A subjected to 4 mol kg^{-1} TFE, which is indicative of a molten globule state at this molality of the alcohol.

To check the difference in conformations, the intrinsic fluorescence of the protein under similar experimental conditions as stated above was studied. Figure 8 presents the intrinsic fluorescence of concanavalin A at different molalities of TFE at pH 2.5. In the absence of TFE the fluorescence spectra of the protein gives the characteristic λ_{max} of concanavalin A at 343 nm. In the presence of 4.0 mol kg^{-1} TFE, there is a red shift to 348 nm beyond which there is a slight red shift to 349 nm along with a decrease in the fluorescence intensity. To further investigate the TFE-induced partially folded conformations, acryl amide quenching experiments were performed. For dynamic or collisional quenching, the process is governed by the equation

$$F_0/F = 1 + K_{SV}[Q] \qquad (6)$$

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. In

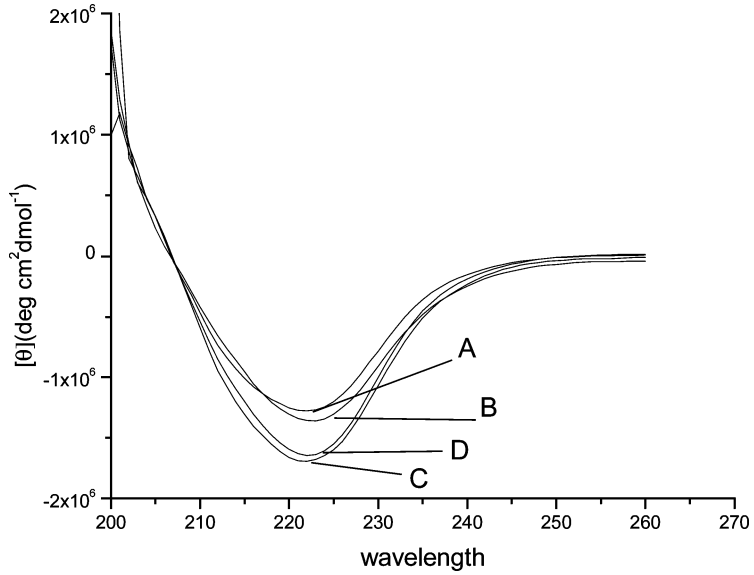

Figure 2. Far-UV CD spectra of 5×10^{-6} mol dm^{-3} of concanavalin A at pH 5.2 at different molalities of TFE: (A) 0, (B) 1, (C) 3, and (D) 5.0 mol kg^{-1}.

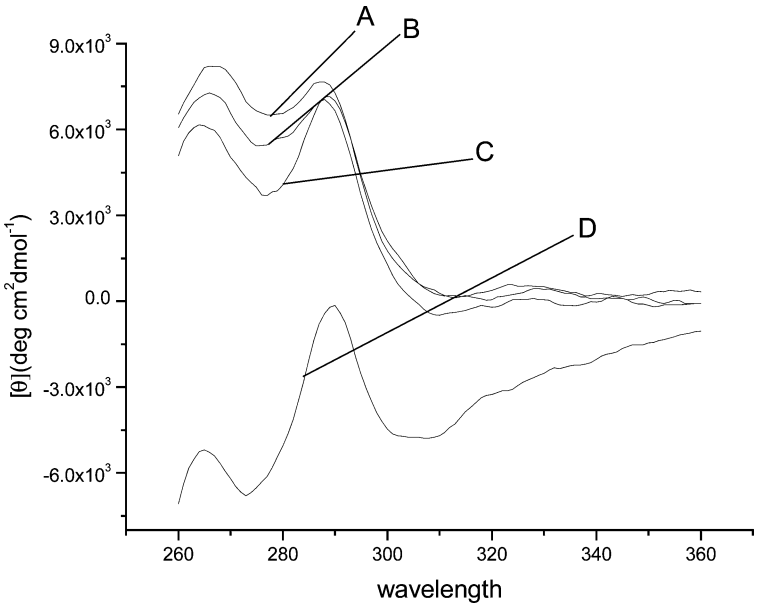

Figure 3. Near-UV CD spectra of 5×10^{-6} mol dm^{-3} of concanavalin A at pH 5.2 at different molalities of TFE: (A) 0, (B) 1, (C) 3, and (D) 5.0 mol kg^{-1}.

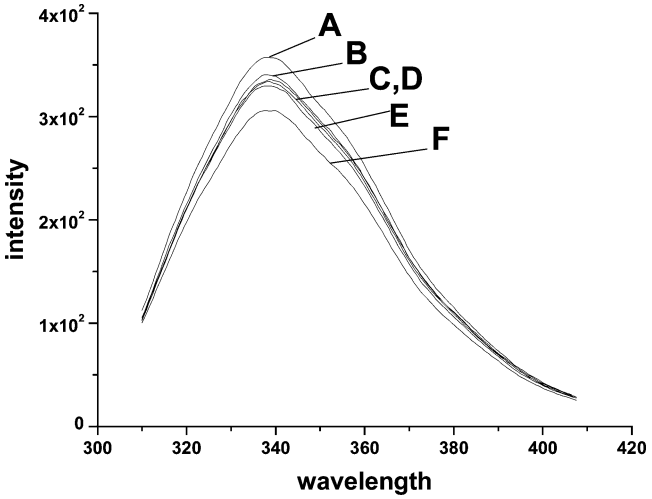

Figure 4. Emission spectrum of 1.8×10^{-6} mol dm^{-3} concanavalin A at different TFE molalities at pH 5.2: (A) buffer, (B) 0.1, (C) 2.0, (D) 3.5, (E) 4, and (F) 5 mol kg^{-1}.

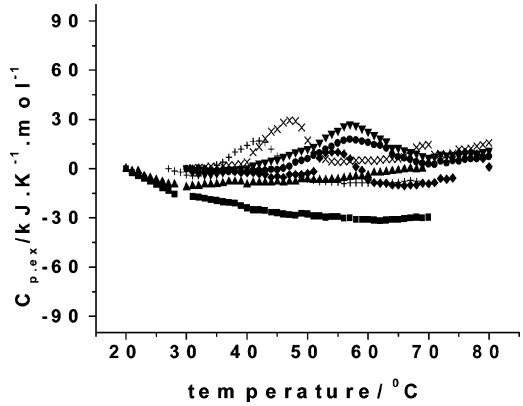

Figure 5. Thermal scans of 0.19×10^{-3} mol dm^{-3} concanavalin A at pH 2.5 in the presence of varying molalities of TFE: (▼) 0, (●) 0.1, (◆) 1.0, (×) 2.0, (+) 3.0, (▲) 3.5, and (■) 4.0 mol kg^{-1}.

presence of 4.0 mol kg^{-1} TFE, the K_{SV} value (5.06 mol kg^{-1}) is between that for the native (1.57 mol kg^{-1}) and denatured form (11.90 mol kg^{-1}) of the protein. ANS binding and energy transfer studies were also performed and are shown in Figures 8 and 9. It was observed that the ANS intensity changes very slightly on addition up to 3.5 mol kg^{-1} TFE but shows maximum intensity in the presence of 4 mol kg^{-1} TFE. The λ_{max} of the ANS intensity also shifts from 510 to 480 nm.

The enhanced secondary structure of concanavalin A at pH 2.5 in the presence of 4.0 mol kg^{-1} TFE and the loss of tertiary structure indicates that concanavalin A is in the molten globule

TABLE 2: Thermodynamic Parameters Accompanying the Thermal Unfolding of 0.19×10^{-3} mol dm^{-3} Concanavalin A at pH 2.5 in Presence of TFE at a Scan Rate of 0.5 K min^{-1} [a]

TFE (mol kg^{-1})	$T_{1/2}$ (K)	ΔH_{cal} (kJ mol^{-1})
0.0	330.8	279
0.1	330.2	270
1.0	329.1	226
2.0	320.4	212
3.0	314.0	195

[a] When errors in sample preparation, reproducibility, and sample impurities are incorporated, the errors in the values of $T_{1/2}$ and ΔH_{cal} are ±0.1 K and 2%, respectively.

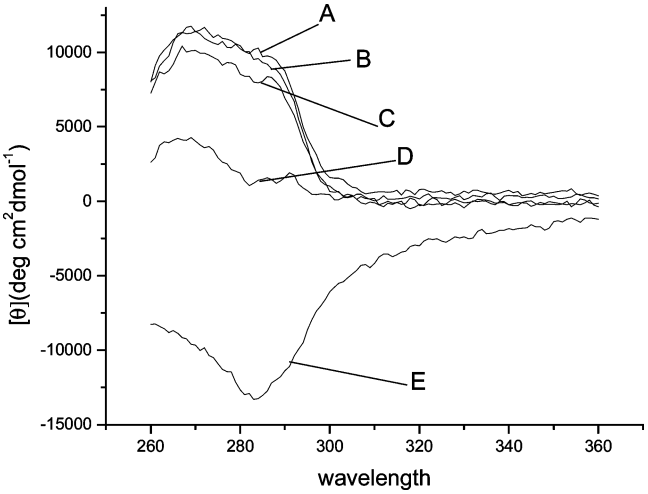

Figure 6. Near-UV CD spectra of 5×10^{-3} mol dm^{-3} of concanavalin A at pH 2.5 at different molalities of TFE: (A) 0, (B) 1.0, (C) 3.0, (D) 4, and (E) 5 mol kg^{-1}.

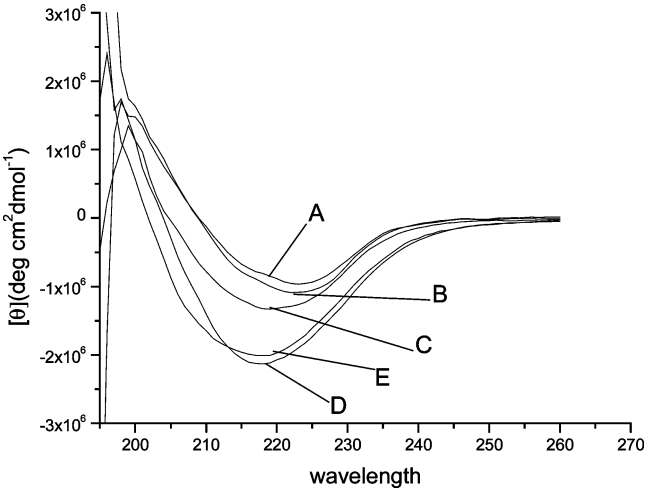

Figure 7. Far-UV CD spectra of 5×10^{-3} mol dm^{-3} of concanavalin A at pH 2.5 at different molalities of TFE: (A) 0, (B) 1.0, (C) 3.0, (D) 4, and (E) 5 mol kg^{-1}.

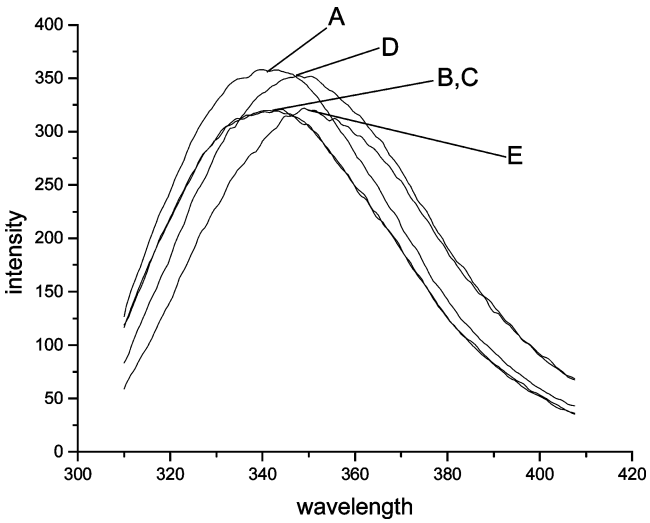

Figure 8. Emission spectrum of 1.8×10^{-6} mol dm^{-3} concanavalin A at different TFE molalities at pH 2.5: (A) 0, (B) 2.0, (C) 3.5, (D) 4, and (E) 5 mol kg^{-1}.

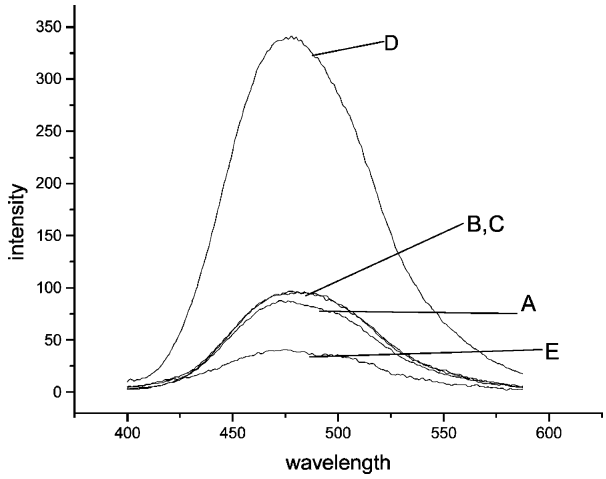

Figure 9. Fluorescence intensity change of 6×10^{-5} mol dm^{-3} ANS upon binding to 2×10^{-6} mol dm^{-3} concanavalin A at pH 2.5: (A) 0, (B) 2.0, (C) 3.5, (D) 4, and (E) 5 mol kg^{-1}.

state under these conditions. Characteristically, MG states have a λ_{max} in between that of the fully native and fully denatured state.[32] Our experimental results also show that at pH 2.5 in 4 mol kg^{-1} TFE the value of λ_{max} is between 343 nm (corresponding to fully native protein) and 354 nm (corresponding to fully denatured protein). Formation of the MG state is also supported by the quenching of fluorescence. The extent of quenching and hence the K_{SV} value depend on the degree to which the quencher achieves the encounter distance of the fluorophore. The Stern−Volmer quenching constant for the native protein was found to be 1.57 mol kg^{-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 nearly 7 times higher (11.9 mol kg^{-1}) than that of the native protein. These values suggest that in the native state the tryptophans are shielded from the quencher to the highest degree and in the denatured state to the least amount. In presence of 4 mol kg^{-1} TFE, the quenching constant is between that of the native and that of the denatured state (5.06 mol kg^{-1}), suggesting an intermediate conformation.

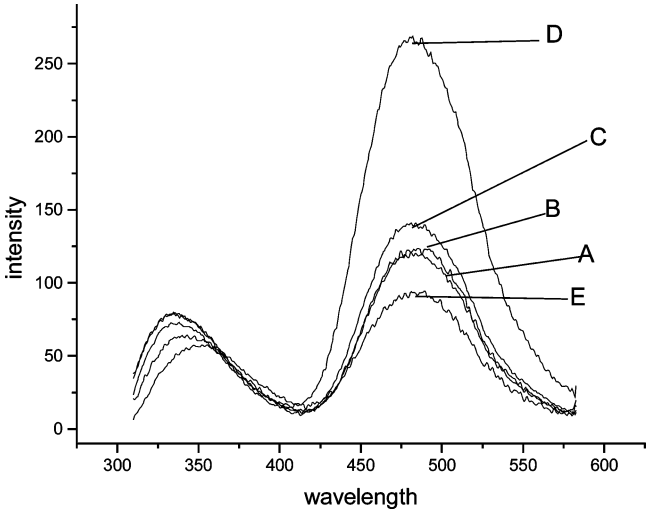

Figure 10. Energy transfer plots between tryptophan of 2×10^{-6} mol dm^{-3} concanavalin A at pH 2.5 and 6×10^{-6} mol dm^{-3} ANS in the presence of (A) 0, (B) 2, (C) 3.5, (D) 4, and (E) 5 mol kg^{-1}.

The hydrophobic dye, ANS, is sensitive to the polarity of its microenvironment; upon binding to the apolar surface, not only is its emission maximum shifted to shorter wavelengths but the emission intensity also is enhanced.[33] Results show that ANS binding to the protein is maximum at 4 mol kg^{-1} TFE. This may be attributed to the presence of a large number of solvent-accessible nonpolar clusters in the presence of 4 mol kg^{-1} TFE. 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 loose their fluorescence intensity, and the ANS fluorescence gains in intensity. From the plots for ANS binding studies (Figure 9), it is observed that the fluorescence increases gradually and in the presence of 4 mol kg^{-1} TFE is maximum. There is a shift of the λ_{max} of ANS fluorescence from 510 nm, corresponding to free ANS, to 470 nm, corresponding to protein-bound ANS.[34] From the plots of energy transfer (Figure 10) in the presence of TFE, 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 tryptophan to bound ANS.

3.3. Binding of ANS with the Native Versus Molten Globule States of Concanavalin A at pH 2.5. Figure 11 shows the isothermal titration calorimetric thermograms of the raw signals obtained from titrating 7.5×10^{-3} mol dm^{-3} ANS with 0.15×10^{-3} mol dm^{-3} concanavalin A in the native and molten

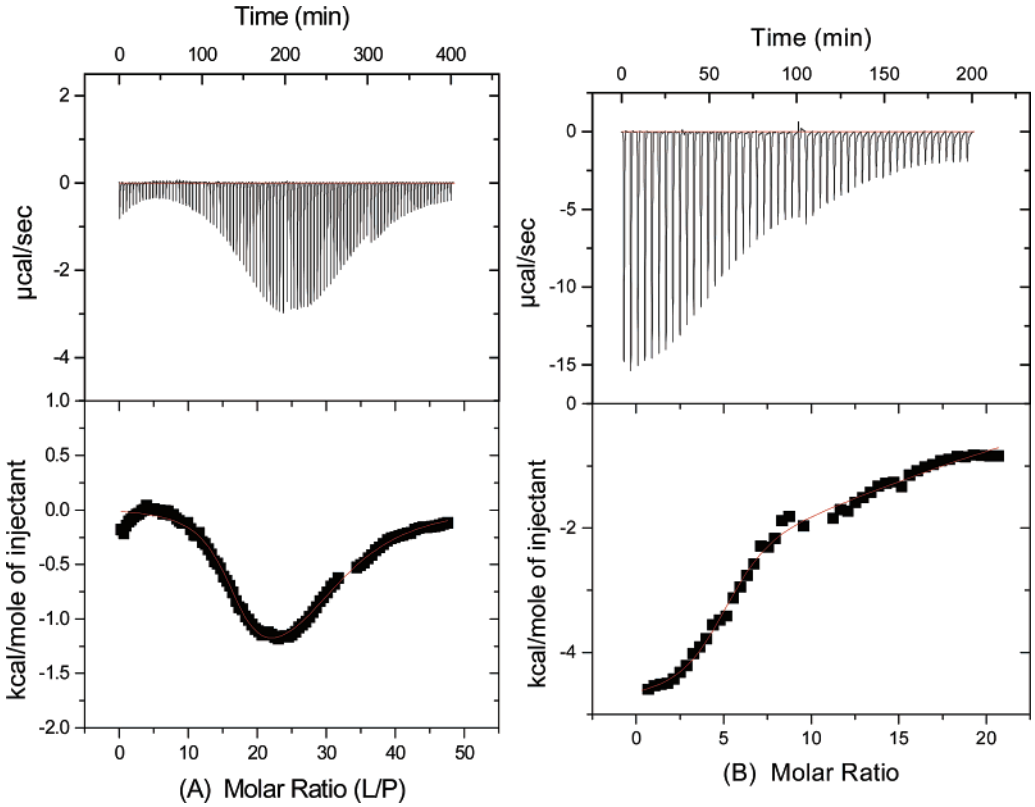

Figure 11. Titration of 7.5×10^{-3} mol dm^{-3} ANS with (A) 0.15×10^{-3} and (B) with 0.15×10^{-3} mol dm^{-3} concanavalin A in the presence of 4 mol kg^{-1} TFE at pH 2.5 and 298.15 K showing the calorimetric response as successive injections of ligand are added to the reaction cell.

TABLE 3: Thermodynamic Parameters for Binding of ANS to Concanavalin A in the Absence and Presence of 4 mol kg^{-1} TFE at pH 2.5 at 298.15 K

TFE (mol dm^{-3})	K_1 (M^{-1})	n_1	ΔH_1 (cal mol^{-1})	ΔS_1 (cal K^{-1} mol^{-1})	K_2 (M^{-1})	n_2	ΔH_2 (cal K^{-1} mol^{-1})	ΔS_2 (cal mol^{-1})
0	$(5.1 \pm 1.5) \times 10^5$	3.84	-382.3 ± 80.7	24.8	$(9.3 \pm 1.1) \times 10^3$	7.15	-3620 ± 149	6.0
4	$(3.28 \pm 2.13) \times 10^3$	14.9	-48.6 ± 6.78	8.9	$(1.33 \pm 0.62) \times 10^4$	5.2	-4845.0 ± 57.0	7.2

globule states of the protein, respectively. Integration of the area of cell feedback by subtracting the dilution heats of both the ligand and the protein gives the differential curves that are shown in the bottom panels of Figure 11. These figures show the amount of heat generated per injection as a function of the molar ratio of ANS to protein.

The binding isotherms show best nonlinear fitting to two independent binding sites. Each site binds to more than one ANS molecule represented by

$$\text{conA} + n_1\text{ANS} = [\text{conA}\cdot n_1\text{ANS}] \quad (7)$$

$$\text{conA}\cdot n_1\text{ANS} + n_2\text{ANS} = [n_2\text{ANS}\cdot\text{conA}\cdot n_1\text{ANS}] \quad (8)$$

where n_1 and n_2 represent the number of moles of ANS molecules binding to the first and second set of sites, respectively. As seen in the bottom panels of Figure 11, the fitting curves (solid lines) show good agreement with experimental data represented by the square symbols. The thermodynamic parameters extracted from the model fitting are summarized in Table 3. This table shows that the binding of ANS molecules to both the binding sites is exothermic and entropy change, though small, is positive. This suggests that the complexation of ANS with the native and molten globule states of the proteins is both enthalpically and entropically favored. The binding of ANS to the native protein is stronger at the first site compared to that at the second site. The reduction in the binding strength at the second site, though $n_2 > n_1$, is due to relatively lesser favorable entropy of binding.

Comparison of the thermodynamic parameters of ANS binding to the acid-induced and TFE-induced molten globule state at pH 2.5 indicates that the binding constant at the first site decreases in the latter. This is due to reduced exothermicity and a favorable entropic contribution. Whereas for the second site the binding constant increases in the MG state, the reaction is more exothermic compared to that at pH 2.5 induced state of the protein, but the value of ΔS does not change appreciably, suggesting that the enhanced binding at the second site is enthalpically driven. In the presence of TFE, the conformation of the protein changes, resulting in further exposure of hydrophobic residues to the solvent environment. This leads to enhanced hydrophobic interactions with the ANS molecules.

On the basis of thermodynamic parameters, some insights into the factors that contribute to the binding of ANS to the molten globule state of conA can be obtained. In principle, a variety of noncovalent forces such as hydrophobic interactions, electrostatic interactions, van der Waals interactions, and hydrogen bonding interactions are responsible for the binding. The reduction in the values of the binding constant at the first site from $(5.1 \pm 1.5) \times 10^5$ to $(3.3 \pm 2.1) \times 10^3$ is due to conformational change that takes place upon addition of TFE. Though the value of n_1 increases from 3.8 to 14.9, the favorable enthalpy of interaction and entropy of binding fall, indicating reduction in the binding ability at this site. The increase in the binding constant at the second site from $(9.3 \pm 1.1) \times 10^3$ to $(1.3 \pm 0.6) \times 10^4$ indicates stronger interaction of the ANS molecules at this site as also reflected in the more negative value

TABLE 4: Ionic Strength Dependence of the Thermodynamic Parameters for Binding of ANS to Concanavalin A in the Presence of 4 mol kg^{-1}TFE at pH 2.5 and 298.15 K

NaCl (mol dm^{-3})	K_1 (M^{-1})	ΔH_1 (cal mol^{-1})	ΔS_1 (cal K^{-1} mol^{-1})	K_2 (M^{-1})	ΔH_2 (cal mol^{-1})	ΔS_2 (cal K^{-1} mol^{-1})
0.20	$(2.10 \pm 0.40) \times 10^3$	-11800 ± 460	-24.4	$(1.01 \pm 0.25) \times 10^3$	-899 ± 205	10.7
0.25	$(3.02 \pm 0.66) \times 10^3$	-14860 ± 5750	-33.9	$(0.89 \pm 0.17) \times 10^3$	-3099 ± 843	3.1
0.30	$(1.48 \pm 0.44) \times 10^3$	-12070 ± 2490	-12.6	$(0.47 \pm 0.10) \times 10^3$	-4869 ± 142	7.2

of ΔH. The negative values of ΔH and positive (though small) values of ΔS are consistent with the characteristics of a combination of van der Waals, hydrophobic, and electrostatic interactions in the binding process. The fluorescence experiments indicate enhanced binding of the ANS molecules to the MG state of concanavalin A compared to the pH 2.5 induced state (Figure 8). Correlation of the fluorescence and calorimetric results indicates that the enhanced binding in the MG state of concanavalin A is essentially due to alteration of the second binding site due to conformational changes in the protein in the presence of TFE.

On the basis of crystallographic data, it has been postulated that the concanavalin A protomer has a site where the hydrophobic molecules can bind.[35,36] This hydrophobic cavity is surrounded by the amino acid side chains of Tyr 54, Leu 81, Leu 85, Val 89, Val 91, Phe 111, Ser 113, Val 179, Ile 181, Phe 191, Phe 212, and Ile 214.[35,36] If this cavity is assumed to be the first binding site, then an increase in ionic strength is not expected to alter the binding affinity of the ligand appreciably. Our results show that the increase in ionic strength does not affect the binding affinity of ANS at the first site significantly (Table 4). This indicates that the binding at this site is mainly via hydrophobic interactions. The increase in the value of stoichiometry of binding to the molten globule state of the protein indicates that TFE induces conformational change, resulting in more accommodation of ANS molecules. Since the value of n_1 in the molten globule state is significantly large, the possibility of other nonspecific sites cannot be excluded.

The value of K_2 falls with an increase in ionic strength (Table 4). This indicates that electrostatic interactions play a major role in the binding of ANS to the molten globule state of concanavalin A at the second site. ANS carries one unit of negative charge, and concanavalin A at pH 2.5 is positively charged (below its isoelectric point). Therefore the presence of salt in solution would act to screen the electrostatic attraction, and hence the affinity of the protein in the molten globule state for ANS decreases with the increase in ionic strength. This indicates that the electrostatic interactions are relevant in binding at the second site.

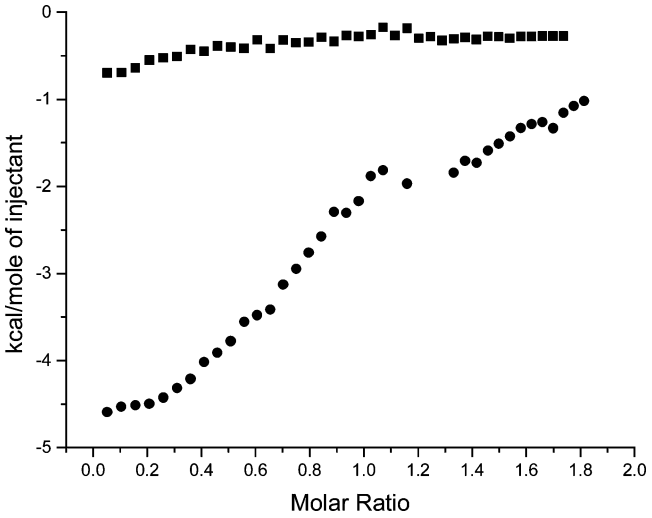

Figure 12. Comparison of titrations of 7.5×10^{-3} mol dm^{-3} ANS with 0.15×10^{-3} mol dm^{-3} concanavalin A in the presence of 4 mol kg^{-1} TFE at pH 2.5 (●) and 5.2 (■) at 298.15 K.

Comparison of the results on ANS binding to concanavalin A in the molten globule state and that at pH 5.2 in the presence of TFE (Figure 12) indicates that the ANS binding to the protein is substantially stronger in the molten globule state.

The pattern in the ITC profile for the binding of ANS to the molten globule state of the protein is different than that with the pH 2.5 acid-induced conformational state (Figure 11). Our results also demonstrate that ITC can be used to characterize the partially folded states of the protein both qualitatively and quantitatively.

4. Conclusions

The differential scanning calorimetrically studied thermal unfolding of concanavalin A in the presence of TFE has permitted the determination of quantitative thermodynamic parameters accompanying all of the transitions and establishment of the mechanism of unfolding. Circular dichroism and fluorescence spectroscopic results corroborate the DSC observations and indicate a protein conformation with a poorly defined tertiary structure and high content of secondary structure in the presence of 4 mol kg^{-1} TFE. This study for the first time demonstrates the thermal unfolding of concanavalin A in the presence of TFE and a partially folded state in the lectin. Isothermal titration calorimetric measurements have demonstrated that ANS binding to the acid-induced state and the partially folded state of concanavalin A displays heterogeneity with two classes of non-interacting binding sites. The association of ANS with concanavalin A in the molten globule state appears to have contributions from hydrophobic interactions and electrostatic interactions that show ionic strength dependence. Our results also demonstrate that ITC can be used to characterize the partially folded states of the protein both qualitatively and quantitatively.

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References and Notes

(1) Levinthal, C. *J. Chim. Phys.* **1968**, *65*, 44.
(2) Edwin, F.; Sharma, Y. V.; Jagannadham, M. V. *Biochem. Biophys. Res. Commun.* **2002**, *290*, 1441.
(3) Kamatari, Y. O.; Konno, T. M.; Kataoka, M.; Akasaka, K. *J. Mol. Biol.* **1996**, *259*, 512.
(4) Fink, A. L.; Calciano, L. J.; Goto, Y.; Kurotsu, T.; Palleros, D. R. *Biochemistry* **1994**, *33*, 12504.
(5) Thomas, P. D.; Dill, K. A. *Protein Sci* **1993**, *2*, 2050.
(6) Shiraki, K.; Nishikawa, K.; Goto, Y. *J. Mol. Biol.* **1995**, *245*, 180.
(7) Liu, Y.; Bolen, D. W. *Biochemistry* **1995**, *34*, 12884.
(8) Schonbrunner, N.; Wey, J.; Engels, J.; Georg, H. *J. Mol. Biol.* **1996**, *260*, 432.
(9) Nelson, J. W.; Kallenbach, N. R. *Biochemistry* **1989**, *28*, 5256.
(10) Buck, M.; Schwalbe, H.; Dobson, C. M. *Biochemistry* **1995**, *34*, 13219.
(11) Hamada, D.; Kuroda, Y.; Tanaka, T.; Goto, Y. *J. Mol. Biol.* **1995**, *254*, 737.

(12) Alexandrescu, A. T.; Ng, Y. L.; Dobson, C. M. *J. Mol. Biol.* **1994**, *235*, 587.
(13) Forge, V.; Wijesinha, R. T.; Balbach, J.; Brew, K.; Robinson, C. V.; Redfield, C.; Dobson, C. M. *J. Mol. Biol.* **1999**, *288*, 673.
(14) Eaton, W. A.; Munoz, V.; Hagen, S. J.; Jas, G. S.; Lapidus, L. J.; Henry, E. R.; Hofrichter, J. *Annu. Rev. Biophys. Biomol. Struct.* **2000**, *29*, 327.
(15) Chakraborty, S.; Peng, Z. Y. *J. Mol. Biol.* **2000**, *298*, 1.
(16) Demarest, S. J.; Fairman R.; Raleigh, D. P. *J. Mol. Biol.* **2000**, *283*, 279.
(17) Peng, Z. Y.; Kim, P. S. *Biochemistry* **1994**, *33*, 2136.
(18) Kuwajima, K. *Proteins* **1989**, *6*, 87.
(19) Ptitsyn, O. B.; Pain, R. H.; Semisotnov, G. V.; Zerovnik, E.; Razgulyaev, O. I. *FEBS Lett.* **1990**, *262*, 20.
(20) Ptitsyn, O. B. *Adv. Protein Chem.* **1995**, *47*, 83.
(21) Shiraki, K.; Nishikawa, K.; Goto, Y. *J. Mol. Biol.* **1995**, *245*, 180.
(22) Beckman, R. P.; Mizzen, L. A.; Welch, W. J. *Science* **1990**, *248*, 850.
(23) Martin, J.; Langer, T.; Boteva, R.; Schramel, A.; Hartl, F. U.; Hoewich, A. L. *Nature* **1991**, *352*, 36.
(24) van der Goot, F. G.; Lakey J. H.; Pattus, F. *Trends Cell Biol.* **1992**, *2*, 349.
(25) Sonnichsen, F. D.; Van Eyk, J. E.; Hodges, R. S.; Sykes, B. D. *Biochemistry* **1992**, *31*, 8790−8798.
(26) McKenzie, C. H.; Sawyer, W. H.; Nichol, L. W. *Biochim. Biophys. Acta* **1972**, *263*, 283.
(27) Kalb, A. J.; Lustig, A. *Biochim. Biophys. Acta* **1968**, *168*, 366.
(28) Bhattacharyya, L.; Koenig, S. H.; Brown, R. D., III; Brewer, C. F. *J. Biol. Chem.* **1991**, *266*, 9835.
(29) Banerjee, T.; Kishore, N. *Thermochim. Acta* **2004**, *411*, 1959.
(30) Becker, J. W.; Reeke, G. N.; Wang, J. L.; Cunningham, B. A.; Edelman, G. M. *J. Biol. Chem.* **1975**, *250*, 1513.
(31) Kirchoff, W. H. *EXAM*; Thermodynamics Division, National Institute of Standards and Technology, U. S. Department of Energy: Gaithersburg, MD, 1988.
(32) Demarest, S. J.; Boice, J. A.; Fairman, R.; Raleigh, D. P. *J. Mol. Biol.* **1999**, *294*, 213.
(33) Semisotnov, G. V.; Rodionova, N. A.; Razgulyaev, O. I.; Uversky, V. N.; Gripaoe, A. F.; Gilmanshin, R. I. *Biopolymers* **1991**, *31*, 119.
(34) Rawitch, A. B.; Hwan, R. Y. *Biochem. Biophys. Res. Commun.* **1979**, *91*, 1383.
(35) Edelman, G. M.; Cunningham, B. A.; Reeke, G. N., Jr.; Becker, J. W.; Waxdal, M. J.; Wang, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 2580.
(36) Becker, J.; Reeke, G. N., Jr.; Wang, J. L.; Cunningham, B. A.; Edelman, G. M. *J. Biol. Chem.* **1975**, *250*, 1513.