Unfolding of the Molten Globule State of α-Lactalbumin Studied by ^{1}H NMR

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ABSTRACT: The urea-induced unfolding of the molten globule state of bovine α-lactalbumin was investigated by ^{1}H nuclear magnetic resonance. In the molten globule state, most of the aromatic resonances deviate from their random coil values, indicating that aromatic side chains form some ordered structures in the molten globule state. When the urea concentration increases, the resonances are shifted, and the deviations from the random coil values are diminished. Because the chemical shifts of several random coil peptides are found to be independent of urea concentration, the urea-induced shifts of the resonances in the molten globule state reflect the unfolding transition of some ordered structures. The unfolding transitions measured by individual aromatic resonances do not coincide with each other. The unfolding transition curves obtained from some aromatic resonances are also different from those of the secondary structures measured by circular dichroism spectra. These results clearly show that the unfolding of the molten globule state of α-lactalbumin is not a cooperative two-state process.

Recently, evidence from a number of experiments has suggested that the molten globule state is a class of intermediates generally observed, at equilibrium or kinetically, in the folding of globular proteins (Kuwajima, 1989; Ptitsyn *et al.*, 1990; Christensen & Pain, 1991; Elöve *et al.*, 1992; Barrick & Baldwin, 1993). Common characteristics of the molten globule state are its compactness and the presence of a significant amount of secondary structure (Kuwajima, 1989; Ptitsyn, 1992). However, its three-dimensional structure has not yet been elucidated in detail, and interactions responsible for the formation of the molten globule state are not understood. Knowledge of these interactions is essential to understand the principle of protein folding and to predict the native three-dimensional structures of proteins from their amino acid sequences.

The importance of hydrophobic interactions has been assumed from the compact globularity of the molten globule state (Kuwajima, 1989; 1992; Dill, 1990; Goto *et al.*, 1990a,b). For the molten globule states of various proteins, anions have been known to be necessary to shield the electrostatic repulsion at acidic pH and to stabilize the molten globule state, otherwise the molecule expands accompanied by the destabilization of secondary structure (Ikeguchi & Sugai, 1989; Goto *et al.*, 1990a,b). These observations indicate the existence of some contractive forces as counterparts of the electrostatic repulsion. Hughson *et al.* (1991) suggested in their study with site-directed mutagenesis that the helical structure in the molten globule state of apomyoglobin is stabilized by nonspecific hydrophobic interactions. If hydrophobic contacts, even if they are nonspecific, stabilize the molten globule conformation, the heat capacity is expected to increase on unfolding of the molten globule. While the heat capacity increment was observed for the molten globule states of cytochrome *c* (Potekhin & Pfeil, 1989; Kuroda *et al.*, 1992) and retinol-binding protein (Bychkona *et al.*, 1992), it was not detected for the molten globule state of apomyoglobin (Griko *et al.*, 1988). The difference in heat capacity between the molten globule and unfolded states of α-lactalbumin is controversial (Xie *et al.*, 1991, 1993; Yutani *et al.*, 1992).

In order to clarify such a complicated situation for our understanding of the stabilization factor of the molten globule state, it is necessary to evaluate the stability of the molten globule conformation with reliable accuracy. Although we have estimated the stability of the molten globule state of α-lactalbumin (the A state) by assuming that its unfolding is a two-state transition (Ikeguchi et al., 1986, 1992; Ikeguchi & Sugai, 1989), the validity of this approximation has not yet been proved. In this study, therefore, we questioned whether or not the unfolding of the A state could be approximated as a two-state transition. NMR[1] is useful for this purpose because it has the potential to monitor the unfolding of individual parts of the molecule. If the unfolding transitions obtained by the resonances of various parts of the molecule coincide with each other, this supports the validity of the two-state approximation. On the contrary, if noncoincidence of the transition curves is observed, it is evidence that the unfolding of the A state is not a two-state transition.

MATERIALS AND METHODS

Materials. Bovine α-lactalbumin (BLA) was prepared from unpasteurized milk. The tetrapeptides, GGHA and GGWA, were purchased from Bachem Feinchemikalien AG. The peptide, GGYA, was synthesized on a EXCELL peptide synthesizer (MilliGen/Biosearch) with standard Fmoc chemistry and purified by reverse-phase HPLC (Waters μBondasphere C18–100 Å 19 × 150 mm). D_2O was obtained from Isotec (99.9% D, 99.96% D), Merck (99.95% D), and CEA (99.95% D). DCl (99.5% D) was obtained from Aldrich. NaOD (99.5% D) and TSP were purchased from Merck. Urea was obtained from Nacalai and was deuterated by repeated lyophilization in D_2O.

Urea Concentration Measurements. The urea concentration of a sample was determined from the refractive index (Pace,

[1] Abbreviations: NMR, nuclear magnetic resonance; 1D, one-dimensional; 2D, two-dimensional; DQF-COSY, double-quantum-filtered 2D *J*-correlated spectroscopy; HOHAHA, 2D homonuclear Hartmann-Hahn spectroscopy; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; HPLC, high-performance liquid chromatography; CD, circular dichroism; BLA, bovine α-lactalbumin; Fmoc, *N*-(9-fluorenylmethoxycarbonyl); TSP, 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate; ANS, 1-anilinonaphthalene-8-sulfonate. Amino acid residues are designated by their standard one-letter abbreviations.

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Table I: Chemical Shifts of the Aromatic Ring Proton Resonances of BLA in the A and U States[a]

Tyrosine			
state	residue	C3,5H	C2,6H
A	103[b]	6.36	6.51
	others	6.73–6.76	6.96–7.02
U	103	6.64	6.80
	others	6.79–6.82	7.06–7.09
random coil		6.84[c]	7.14[c]
		6.86[d]	7.15[d]

Tryptophan							
state	residue	N1H	C2H	C4H	C5H	C6H	C7H
A[g]	104[b]	10.02[e]	7.21[e]	7.49	6.96	7.08	7.39
	U1	9.91[e,f]	7.17[e,f]	7.49	6.96	7.02	7.32
	U2	9.97[e,f]	7.16[e,f]	7.36	6.87	7.02	7.32
	U3	10.00[e]	7.19[e]	7.41	6.91	7.04	7.37
U	104	nd	nd	7.59	7.10	7.20	7.46
	U1	nd	nd	7.52	7.10	7.20	7.46
	U2	nd	nd	7.48	7.06	7.16	7.42
	U3	nd	nd	7.53	7.07	7.17	7.43
random coil		nd	7.25[c]	7.65[c]	7.17[c]	7.25[c]	7.51[c]
		10.22[d]	7.24[d]	7.65[d]	7.17[d]	7.24[d]	7.50[d]

Histidine			
state	residue	C2H	C4H
A	32	8.56	7.20
	68	8.63	7.33
	107	8.42	7.02
U	32	8.58	7.21
	68	8.64	7.32
	107	8.52	7.18
random coil		8.61[c]	7.32[c]
		8.61[d]	7.35[d]

[a] The values (in ppm) were measured at pH 2 and 35 °C in the absence and presence of 10.4 M urea for the A and U states, respectively. U1, U2, and U3 denote unassigned tryptophanyl residues. nd means not determined. [b] The assignments are based on Alexandrescu *et al.* (1993). [c] The values were measured for the tetrapeptides at pH 2 and 35 °C. [d] The values are from Wüthrich (1986) and Bundi and Wüthrich (1979). The values of histidine are those of the protonated form. [e] The values were obtained in 90% H_2O/10% D_2O. [f] The values of U1 and U2 are possible to be reversed, because the C7H resonances, with which the N1H resonances were connected by NOEs, are overlapped. [g] The values were measured at 45 °C because of poor quality of the spectrum at 35 °C. For these resonances, however, there was little difference in the chemical shifts between 35 and 45 °C.

1986). We assumed that the difference in the refractive index between a deuterated urea solution and D_2O is identical with that between a urea solution and H_2O.

NMR Measurements. All NMR experiments were performed with a Jeol A-500 spectrometer. All spectra were recorded over a spectral width of 8000 Hz. Chemical shifts were measured relative to an internal standard, TSP. However, for measurements of the protein, an impurity peak at 0.148 ppm was used as standard, because the resonance of TSP was found to shift depending on the protein concentration, probably due to the interaction with the protein (A. Shimizu, M. Ikeguchi, and S. Sugai, manuscript in preparation). The resonance frequency of the impurity was independent of the protein concentration. All 1D spectra were measured with a digital resolution of 0.49 Hz. DQF-COSY (Rance *et al.*, 1983), HOHAHA (Bax & Davis, 1985) and NOESY (Jeener *et al.*, 1979; Kumar *et al.*, 1980) spectra were acquired in the phase-sensitive mode with the TPPI-States method (Marion et al., 1989). The 2D exchange correlation experiments were carried out using the NOESY pulse sequence. For all 2D experiments, data sets resulting from 256–512 t_1 increments of 1024 data points were collected and zero-filled to achieve a digital resolution of 3.9 Hz. Spectra were resolution enhanced by shifted sine-bell functions in both dimensions. Solvent suppression was achieved by presaturation. Unless otherwise indicated, the protein concentration was 1 mM. The spectra of the peptides were measured at peptide concentrations of 3 mM. The pD values reported are uncorrected pH meter readings.

CD Measurements. The CD spectra were measured on a Jasco J-720 spectropolarimeter that was calibrated with *d*-10-camphorsulfonic acid. An optical cell with a 1-mm path length was used throughout. The protein concentration was 35–40 μM. The temperature was kept at 35 °C with a water-circulating cell holder.

RESULTS

^1H NMR Characterization of the A State. The first stage assignments in the A state were done by DQF-COSY, HOHAHA, and NOESY experiments. This is relatively easy for the aromatic resonances. The spin systems of ring protons could be identified for four tryptophans (W26, W60, W104, and W118), four tyrosines (Y18, Y36, Y50, and Y103), and three histidines (H32, H68, and H107). The sequence-specific assignments have been made by exchange correlation with the preassigned resonances in the native (N) state (Bradbury & Norton, 1975; Koga & Berliner, 1985; Harushima & Sugai, 1989; Alexandrescu *et al.*, 1992). The 2D exchange correlation experiment (τ_m = 150 ms) was carried out at pD 3.35 and 35 °C, where the N and A states are interconverting. The exchange cross-peaks were observed for the C2H resonances of all histidine residues, making it possible to assign the ring proton resonances of all of these residues (Table I). Our assignments for the resonances of H107 agree with those reported recently (Alexandrescu *et al.*, 1993). Although no exchange correlation could be identified for the other resonances under the conditions used here, the resonances of Y103 and W104 have been assigned according to Alexandrescu *et al.* (1993). The chemical shifts of the aromatic resonances are summarized in Table I. Although the values are slightly different from those reported by Alexandrescu *et al.* (1993),

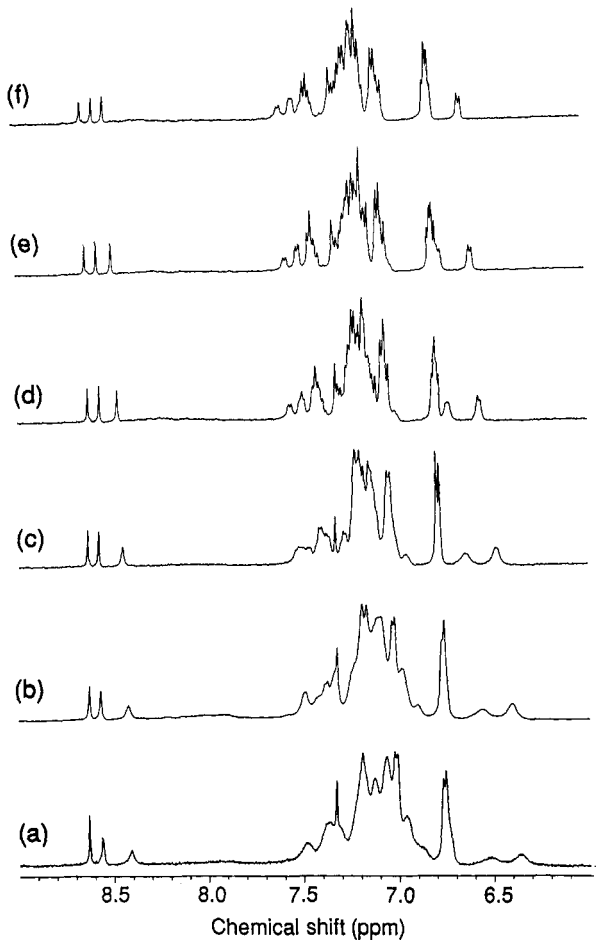

FIGURE 1: Aromatic region of ^{1}H NMR spectra of BLA recorded at 0 (a), 1.87 (b), 3.85 (c), 6.21 (d), 7.69 (e), and 10.38 M (f) urea in D_2O (pD 2, 35 °C).

this difference may be due to the protein concentration dependence of the TSP resonance, which was used as a chemical shift standard by Alexandrescu *et al.* (1993) (see Materials and Methods).

As reported recently (Alexandrescu *et al.*, 1993), most of the ring proton resonances are shifted upfield of the random coil values (Bundi & Wüthrich, 1979; Wüthrich, 1986), suggesting that most of the aromatic side chains are involved in some tertiary interactions in the A state. The ring proton resonances of all tryptophans are shifted 0.1–0.3 ppm upfield of the random coil values. Of the four tyrosines, Y103 shows the largest deviation from the random coil shift (0.5–0.6 ppm). The ring proton resonances of the remaining three tyrosines are shifted by only 0.1–0.2 ppm. The broadness of the peak is also characteristic of Y103. Of the three histidines, H107 shows the largest deviation (0.2–0.3 ppm) from the random coil values and the broadest line width. Although the C2H and C4H resonances of H32 and H68 are shifted upfield or downfield, the deviation from the random coil value is smaller than 0.1 ppm. These results indicate the uniqueness of the 101–110 region, as previously shown (Alexandrescu *et al.*, 1993). The interresidue NOEs observed by Alexandrescu *et al.* (1993) are strong evidence for the cluster formation in this region.

Urea-Induced Shift of the Resonances in the A State. In order to confirm that the perturbed resonances described above reflect the tertiary interaction in the A state, we investigated the urea concentration dependence of the chemical shifts. Shown in Figure 1 are the aromatic regions of ^{1}H NMR spectra

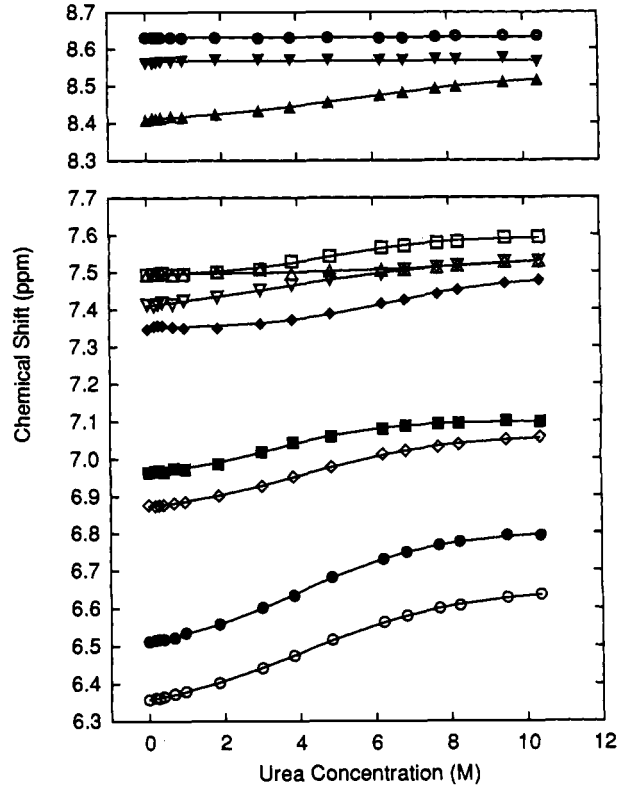

FIGURE 2: Urea concentration dependence of the chemical shifts of some aromatic resonances of BLA (pD 2, 35 °C): Y103 C3,5H (○), Y103 C2,6H (●), WU2 C5H (◇), W104 C5H (■), WU2 C4H (◆), WU3 C4H (▽), WU1 C4H (△), W104 C4H (□), H107 C2H (▲), H32 C2H (▼) and H68 C2H (◎).

at various concentrations of urea. With increasing urea concentration, the spectrum as a whole is shifted to lower field and sharpens up. Resolved resonances such as those of C2,6H and C3,5H of Y103 and C2H of H107 are shifted progressively to lower field. The urea-induced shifts of some overlapped resonances were followed by DQF-COSY, although most of the resonances overlapped in the 1D spectrum are also overlapped in the DQF-COSY spectrum, at least at some urea concentrations, and could not be followed throughout the entire concentration range of urea. Among the cross-peaks between the ring protons of tryptophan, the C4H–C5H cross-peak is relatively well resolved and easy to follow. However, the C4H and C5H resonances of W104 are overlapped with those of another tryptophan, and their urea-induced shifts could not be distinguished by DQF-COSY spectra. The C4H resonance of W104 could be distinguished from the other C4H resonance by the C4H–C7H cross peak in the HOHAHA spectrum (τ_m = 90 ms). The results are shown in Figure 2. All urea-induced shifts are downfield, i.e., toward the random coil shifts. The chemical shifts at 10.4 M urea do not deviate more than 0.1 ppm from the random coil values except for a few resonances (Table I). The dependence of the chemical shifts on urea concentration is sigmoidal; therefore, the urea-induced shift of the resonances seems to indicate the unfolding of structures formed in the A state.

Because the magnitude of the urea-induced shift, however, is generally small, the possibility that the interaction of urea with the protein, especially with the aromatic side chains, produces the shift in resonance must be taken into account. Thus, we have carried out urea titration experiments for model peptides. The model peptides used are GGWA, GGYA, and GGHA, which were used to estimate the random coil shift (Bundi & Wüthrich, 1979; Wüthrich, 1986). The experi-

mental conditions were the same as those used for the protein (pD 2.0, 35 °C). The ring proton resonances of tyrosine, tryptophan, and histidine residues in the peptides are independent of urea concentration (±0.005 ppm). Although the chemical shifts of other resonances of the peptides are slightly altered by urea, their changes are within 0.04 ppm (most are within 0.02 ppm). The resonances that do not deviate from the random coil values in the A state (e.g., the C2H resonances of H32 and H68) are also independent of urea concentration. The independence of the chemical shifts has also been shown for the native state of lysozyme (Lumb & Dobson, 1992). Therefore, the urea-induced shifts of the resonances observed for the A state (Figure 2) are not ascribed to any direct interaction with urea. Furthermore, the possibility that the urea-induced shift arises from protein association is excluded because, at any concentration of urea, 1D spectra measured at 0.5 mM protein agree well with those of 1 mM protein. In conclusion, the urea-induced shifts observed reflect the unfolding of structures in the A state.

Nature of the Unfolding of the A State. In order to compare the urea-induced shifts of the resonances with each other, the urea concentration dependence shown in Figure 2 was normalized by fitting the following equation:

$$\delta_{obs} = \frac{\delta_S + \delta_U \exp(-\Delta G/RT)}{1 + \exp(-\Delta G/RT)} \quad (1)$$

where δ_{obs}, δ_S, and δ_U are the chemical shift observed at a given concentration of urea, that of the structured state, and that of the unstructured state, respectively. R is the gas constant, and T is the absolute temperature. ΔG is the free energy of stabilization of a structure responsible for the deviation of the chemical shift from the random coil value and is assumed to be a linear function of urea concentration:

$$\Delta G = \Delta G^{H_2O} - m[\text{urea}] \quad (2)$$

where ΔG^{H_2O} is the free energy at 0 M urea, and m is the cooperativity parameter (Pace, 1986). The results are shown in Figure 3 and Table II. The unfolding transition curves monitored by various resonances do not coincide with each other. Although the C4H and C5H of W104 and the C4H of an unassigned tryptophan (WU2) show similar cooperativity of transition, the midpoints of their transitions differ from each other by more than 1.5 M. The transition curves of Y103 and the C5H of WU2, which do coincide with each other, show lower cooperativity than the three resonances above. Even lower cooperativity is observed for the transitions of H107 and WU3. These differences in the cooperativity or the midpoint are beyond experimental error. These results indicate that the unfolding of the A state is not a two-state process.

Since the unfolding of the secondary structure in the A state could not be measured by the urea-induced shifts of the ^{1}H NMR peaks, the urea-induced unfolding of the A state was measured by CD and compared with the results from ^{1}H NMR. Figure 4 shows the urea-induced change in the ellipticity at 222 nm ($[\theta]_{222}$) under the same experimental conditions as used for NMR, i.e., at pD 2.0 and 35 °C. D_2O and deuterated urea were used in the experiments, because their effects on the conformational stability may be different from those of H_2O and nondeuterated urea. There is a difference between the results in D_2O and those in H_2O, but it is not significant. Because a linear increase in $[\theta]_{222}$ above 6 M urea has been generally observed at the posttransition region in the denaturant-induced unfolding of proteins and does not result from the disruption of the secondary structure

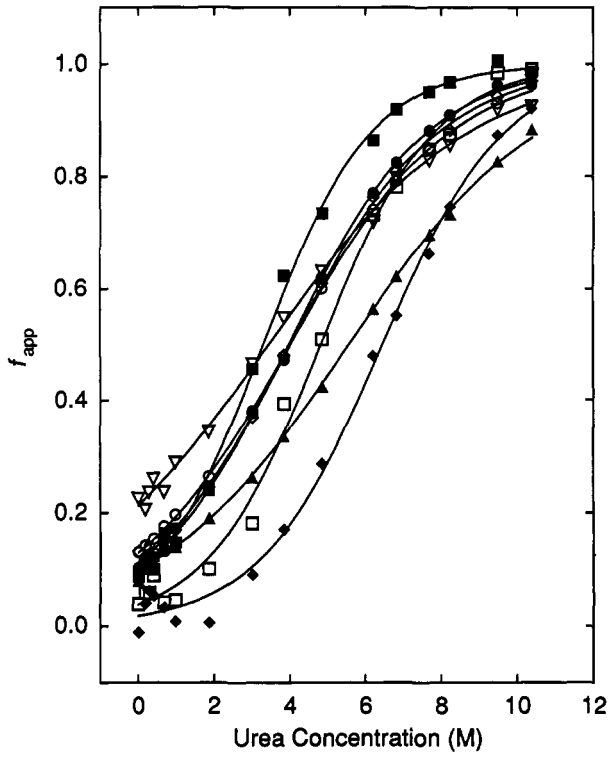

FIGURE 3: Normalized transition curves obtained from the urea-induced shifts of the resonances shown in Figure 2. f_{app} is the apparent fractional extent of changes. Symbols are the same as in Figure 2.

Table II: Fitted Parameters of the Urea-Induced Shifts of Resonances[a]

	δ_S (ppm)	δ_U (ppm)	ΔG^{H_2O} (kcal/mol)	m (kcal/mol/M)	C_m[b] (M)
Y103 C3,5H	6.314	6.650	1.16	0.288	4.01
	(0.003)	(0.002)	(0.04)	(0.007)	(0.16)
Y103 C2,6H	6.479	6.805	1.32	0.333	3.97
	(0.004)	(0.003)	(0.06)	(0.012)	(0.22)
W104 C4H	7.489	7.594	1.97	0.408	4.81
	(0.003)	(0.003)	(0.22)	(0.045)	(0.75)
W104 C5H	6.951	7.100	1.40	0.426	3.30
	(0.005)	(0.002)	(0.15)	(0.035)	(0.45)
WU2 C4H	7.350	7.488	2.44	0.378	6.45
	(0.002)	(0.007)	(0.23)	(0.041)	(0.93)
WU2 C5H	6.849	7.063	1.25	0.311	4.02
	(0.004)	(0.003)	(0.08)	(0.016)	(0.32)
WU3 C4H	7.376	7.540	0.80	0.231	3.44
	(0.013)	(0.005)	(0.18)	(0.032)	(0.91)
H107 C2H	8.398	8.535	1.33	0.239	5.58
	(0.003)	(0.004)	(0.09)	(0.018)	(0.58)

[a] The fitting function and the parameters are described in the text. The values in parentheses are standard errors. [b] The urea concentration at the midpoint of the transition, which was calculated from the fitted values of ΔG^{H_2O} and m.

(Kosen *et al.*, 1981; Vita & Fontana, 1982; Ikeguchi *et al.*, 1986; Ikeguchi & Sugai, 1989; Shortle & Meeker, 1989; Sugawara *et al.*, 1991), the secondary structure in the A state is completely disrupted at 6 M urea. The unfolding of the secondary structure in the A state, therefore, does not coincide with the unfolding of some structures monitored by the ^{1}H NMR signals. For example, the urea-induced shifts of the resonances of Y103, W104, and H107 are not complete at 6 M urea, indicating that the cluster in the 101–110 region is not completely disrupted at 6 M urea (Figure 3). In conclusion, the unfolding of the A state cannot be regarded as a two-state transition.

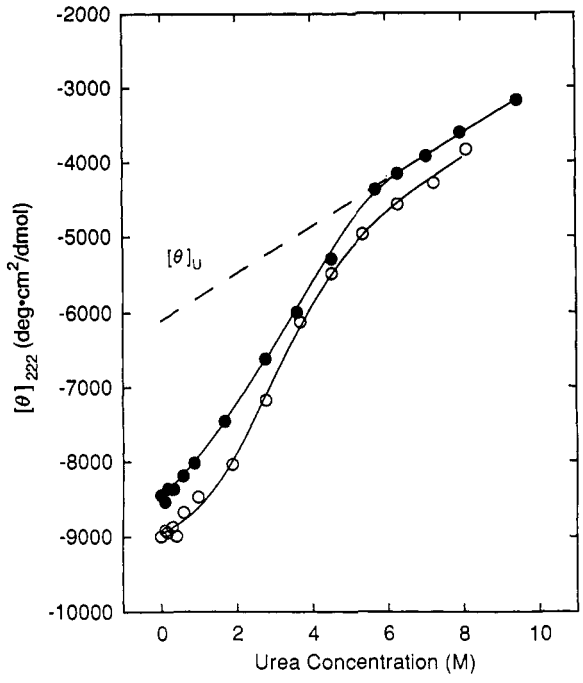

FIGURE 4: Urea concentration dependence of $[\theta]_{222}$ of BLA in D_2O at pD 2 (●) and in H_2O at pH 2 (○). Both sets of data were obtained at 35 °C. The broken line represents the ellipticity of the unfolded state.

DISCUSSION

As shown in this study and in previous work (Baum *et al.*, 1989; Evans *et al.*, 1991; Neli *et al.*, 1992; Alexandrescu *et al.*, 1993; Stockman *et al.*, 1993), the deviation of the chemical shift from the random coil value is an indication of residual structure in the unfolded protein. Neli *et al.* (1992) used the value of 0.1 ppm for nonlabile protons as a threshold by which the chemical shift perturbation indicating residual structure was distinguished from others. The present results support the value of 0.1 ppm as appropriate for the threshold. Of the resonances investigated here, almost all of the resonances that deviate from the random coil values by more than 0.1 ppm in the A state are shifted by urea (Table I and Figure 2). Because the chemical shifts of the random coil peptides do not depend on urea concentration, the occurrence of a urea-induced shift in the resonances of unfolded proteins is strong evidence for the existence of residual structure. The chemical shift perturbation and the urea-induced shift observed for many aromatic resonances in the A state indicate the presence of some tertiary structure in the A state. One such structure has been identified as a cluster in the 101–110 region (Alexandrescu *et al.*, 1993). Because the resonances of two of the three tryptophans other than W104 and the three tyrosines other than Y103 are shifted by urea (Figure 1 and 2), other clusters may be formed in the A state in addition to the cluster in the 101–110 region. Recently, the indole NH hydrogen of W26 was reported to be protected against exchange in the A state of guinea pig α-lactalbumin (Chyan *et al.*, 1993). At 10.4 M urea, few aromatic resonances deviate from the random coil values by more than 0.1 ppm (Table I). A pronounced exception is Y103. The ring proton resonances of Y103 deviate from the random coil values by more than 0.2 ppm, even at 10.4 M urea (Table I). Because the urea concentration dependence of these resonances indicates that the cluster in the 101–110 region is disrupted at 10.4 M urea (Figure 2), the perturbation of these resonances at 10.4 M urea may be attributed to conformation-independent local interactions in this region. Alexandrescu *et al.* (1993) have observed that the perturbation of the Y103 resonances for the peptide corresponds to the 101–110 region in water, where the interresidue NOEs indicative of cluster formation were not detected. Of the ring proton resonances of tryptophan, only the C4H resonance is apt to deviate from the random coil values at 10.4 M urea (Table I). The reason for these deviations is not clear at present.

The unfolding transition curves obtained from the urea concentration dependence of aromatic resonances do not coincide with each other (Figure 3). While some of them apparently coincide with the unfolding of the secondary structure measured by CD, others do not. These results show that the unfolding of the A state is not a cooperative two-state process. This is in contrast to the two-state unfolding shown for the molten globule states of carbonic anhydrase, β-lactamase (Ptitsyn, 1992; Uversky *et al.*, 1992), cytochrome *c* (Goto *et al.*, 1990b; Kataoka *et al.*, 1993), and apomyoglobin (Goto *et al.*, 1990b). In these studies, the two-state transition was shown by various experimental results: the presence of isosbestic, isodichroic, or isoscattering points, the coincidence of the transition curves measured by various methods, or the observation of two peaks corresponding to the molten globule and unfolded states in size exclusion chromatography. Although, for the A state of BLA, the unfolding transitions monitored by various aromatic resonances do not coincide with each other, the transitions occur over a wide concentration range of urea, and the transition zones are considerably overlapped (Figure 3). If this is the case for other proteins, the transition curves monitored by averaged observable parameters, such as ellipticity, viscosity, or scattering intensity, may apparently coincide with each other. The apparent isosbestic, isodichroic, or isoscattering points may be observed for such overlapped transitions. Although the separation of the molten globule and unfolded states by size exclusion chromatography shows that there are two discrete conformational states that differ in molecular size, each conformational state may be an ensemble of several conformations whose NMR spectra differ from each other. Therefore, it remains possible that the unfolding of the molten globule states of other proteins is not a two-state transition. The noncooperativity in the unfolding of the molten globule of cytochrome *c* was observed in D_2O (Jeng & Englander, 1991). More work is needed to clarify whether or not there is a generality in the cooperativity of the molten globule state.

Each resonance in the A state is shifted as a single peak when the urea concentration is increased. Therefore, the unfolding of the structure in the A state is a fast exchange process on the NMR time scale. From the magnitudes of the urea-induced shifts (0.1–0.3 ppm), the rate constant of the interconversion between the structured and unstructured states is estimated to be greater than 500 s^{-1}. Previously, Baum *et al.* (1989) had estimated the rate of interconversion between a number of different conformations of the molten globule to be slower than 1 ms on the basis of the magnetic field dependence of the line widths. This may not be inconsistent with the present results. Because the resonances in the A state became progressively sharper when the urea concentration is increased, the rate of interconversion is thought to be accelerated with increasing urea concentration. Even though the rate is slower than 1 ms in the absence of urea, the acceleration of the rate in the presence of urea must bring the rate into the range of our estimate. Our estimate is also consistent with the rapidity of the formation of the secondary structure indicated by CD stopped-flow experiments (Gilmanshin & Ptitsyn, 1987; Ikeguchi *et al.*, 1992), but is

inconsistent with the rate of compaction measured by the ANS-binding experiment (Ptitsyn *et al.*, 1990). However, no detailed comparison is possible at present, because the relation between the shift of the ^{1}H NMR peak and the compaction is not clear.

Although it has been shown that Y103, W104, and H107 are included in a cluster formed in the A state (Alexandrescu *et al.*, 1993), the unfolding transition curves do not coincide even between these residues (Figure 3). This is apparently inconsistent with the previous observation that, in disulfide-reduced BLA, the ionization of H107 induces chemical shift changes in the ring proton resonances of Y103 at a pH similar to the pK_a of H107 (Alexandrescu *et al.*, 1993). A possible interpretation is that, although the unfolding of the cluster in the 101–110 region can be regarded as a two-state transition, conformational changes in other parts of the molecule, which occur at a more or less different concentration range of urea, affect the chemical shifts of the resonances of the residues within the cluster and produce an apparent noncoincidence of the transition curves. For example, the change in distance or orientation between the residues in the cluster and some aromatic side chains that do not directly interact with the residues in the cluster may result in the change in the ring current effects on the resonances of the residues in the cluster. Although such an influence is expected to be subtle, it may be sufficient to alter the shape of the transition curves of the resonances of W104 and H107, because the magnitude of the urea-induced shifts is small. Whether or not this interpretation is valid may be clarified by our study currently in progress on the unfolding of the cluster formed in the peptide corresponding to the 101–110 region.

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