Conformational States of β-Lactamase: Molten-Globule States at Acidic and Alkaline pH with High Salt†

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ABSTRACT: We present evidence that β-lactamase is close to fully unfolded (i.e., random coil conformation) at low ionic strength at the extremes of pH and that the presence of salt causes a cooperative transition to a conformation with the properties of a molten globule, namely, a compact state with native-like secondary structure but disordered side chains (tertiary structure). The conformation of β-lactamase I from *Bacillus cereus* was examined over the pH 1.5–12.5 region by circular dichroism (CD), tryptophan fluorescence, dynamic light scattering, and 1-anilino-8-naphthalenesulfonate (ANS) binding. Under conditions of low ionic strength (I = 0.05) β-lactamase was unfolded below pH 2.5 and above pH 11.5, on the basis of the far-UV and near-UV CD and tryptophan fluorescence. However, at high ionic strength and low pH an intermediate conformation (state A) was observed, with a secondary structure content similar to that of the native protein but a largely disordered tertiary structure. The transition from the unfolded state (U) to state A induced by KCl was cooperative and had a midpoint at 0.12 M KCl (I = 0.17 M) at pH 1.6. A similar conformation (state B) was observed at high pH and high ionic strength. The transition from the alkaline U state to state B induced by KCl at pH 12.2 was cooperative and had a midpoint at 0.6 M KCl (I = 0.65 M). Light scattering measurements showed that state B was compact although somewhat expanded compared to the N state. The compactness of state A could not be determined due to its strong propensity to aggregate. Both states A and B bound ANS strongly, in contrast to the N and U states, indicating the existence of increased hydrophobic surface. The transitions between states U, A, and N, and U, B, and N, were reversible, although complicated by aggregation of state A and chemically induced damage to states U and B at high pH. The results indicate that hydrophobic interactions play an important role both in the formation of the A and B states from the unfolded state and in their transformation to the native state.

Protein folding is generally a highly cooperative process, in which only native and unfolded states are stable. Recent studies (both equilibrium and kinetic), however, have shown the existence of intermediate conformational states for several proteins (Kim & Baldwin, 1982). Characterization of such intermediate states is an important aspect of the protein folding problem. An understanding of the structural and thermodynamic properties of such states should aid in determining the major factors involved in guiding the pathway for folding.

Of particular interest to the question of protein folding is the observation that in a few cases stable intermediate conformational states have been observed that have compact structures with similar secondary structure content to the native state, but with disordered tertiary structures. The term "molten globule" has been given to these states (Ohgushi & Wada, 1983; Ptitsyn, 1987). Although it has been known for a long time that thermal- or acid-denatured proteins may retain some secondary structure [e.g., Aune et al. (1967) and Tanford (1968)], the first clear-cut examples of a molten-globule state were those of α-lactalbumin under denaturation conditions of either high temperature, acidic pH, or low Gdn·HCl[1] [Dolgikh et al., 1981, 1985; see also Kuwajima (1977) and Kuwajima et al. (1985)] and that of acid-denatured cytochrome *c* (Ohgushi & Wada, 1983). From the experimental point of view the major distinguishing characteristics of the molten-globule state are a far-UV CD spectrum very similar to that of the native state, a near-UV CD spectrum similar to that of the fully unfolded state (e.g., with 6 M Gdn·HCl), a compact structure as measured by intrinsic viscosity or dynamic light scattering, NMR side-chain signals (usually from aromatic residues) that are similar to those of the fully unfolded state, noncooperative thermal melting, and fast H/D exchange (Brazhnikov et al., 1985; Dolgikh et al., 1984, 1985; Ptitsyn, 1987).

The results of kinetics experiments with several proteins have shown the rapid formation of intermediates with properties consistent with those of the molten-globule state, supporting the idea that the molten-globule state may be an obligatory intermediate on the folding pathway (Dolgikh et al., 1984, 1985; Kuwajima et al., 1985; Ikeguchi et al., 1986; Ptitsyn, 1987; Semisotnov et al., 1987). The exact role of molten-globule states in protein folding is still controversial, however (Konishi et al., 1982; Denton et al., 1982; Lynn et al., 1984). As indicated above, evidence for molten-globule states has so far been observed for very few proteins; consequently, it is important to study others in order to determine the generality of such states and to determine their role in protein folding.

β-Lactamase I from *Bacillus cereus* has a MW of 28.5K and has no disulfide bonds. This β-lactamse is similar to that from *Staphylococcus aureus*, whose folding properties have been studied extensively by Pain and co-workers [e.g., Robson and Pain (1976a,b), Carrey and Pain (1978), Mitchinson and Pain (1985), and Craig et al. (1985)], with about 40% amino

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[1] Abbreviations: PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CD, circular dichroism; Gdn·HCl, guanidine hydrochloride.

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acid homology and very similar three-dimensional structures (Samraoui et al., 1986; Herzberg & Moult, 1987). A molten-globule-like state, but with a compactness much greater than that of the native conformation, has been reported for β-lactamase from *S. aureus* in the presence of urea and Gdn·HCl (Robson & Pain, 1976a,b; Mitchinson & Pain, 1985).

The aims of this investigation were to map out the pH-dependent unfolding transitions of β-lactamase I and to characterize the nonnative conformations as a function of ionic strength and pH. Unique conformational states at both acidic and alkaline pH regions were found at high salt concentrations which showed characteristic properties of the molten-globule state. The results indicated that hydrophobic interactions play an important role in the formation of intermediate conformational states during folding.

Materials and Methods

Materials

β-Lactamase I from *B. cereus* strain 569/H/9 was prepared according to the method of Davies and Abraham (1974) and stored in 50 mM sodium phosphate buffer, pH 7.0, at 4 °C. The enzyme was homogeneous by HPLC analysis using an Ultrapore C3 column (Beckman) and by SDS–PAGE. Benzylpenicillin was purchased from Sigma. Gdn·HCl (Ultrapure) was obtained from Schwarz/Mann Biotech; other chemicals were reagent grade and were used without further purification.

Methods

Fluorescence Measurements. Fluorescence was measured with a Perkin-Elmer fluorescence spectrophotometer, Model MPF-4. Tryptophan fluorescence was measured with excitation at 280 nm. The protein concentration was 1 μM. ANS fluorescence was measured with excitation at 365 or 400 nm and with absorbance less than 0.1 absorbance unit at the excitation wavelength.

Rayleigh light scattering experiments were done with the fluorometer to follow protein aggregation. Both the excitation and emission were set to 330 nm, and the change in scattering intensity was followed.

Circular Dichroism Measurements. CD measurements were carried out with a modified Jasco spectropolarimeter, Model J-20. The instrument was calibrated with *d*-10-camphorsulfonic acid. Data were collected at 1-nm intervals with time averaging. The results are expressed as mean residue ellipticity $[\theta]$, which is defined as $[\theta] = 100\theta_{obsd}/(lc)$, where θ_{obsd} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in cm. For calculation of c, a value of 108.5 was used as the mean residue molecular weight. CD spectra were measured at a protein concentration of 20 μM with a 10-mm cell from 320 to 240 nm and with a 0.2-mm cell from 260 to 185 nm. The salt-dependent conformational transition was measured at a protein concentration of 5 μM with a 1-mm path length cell at 220 nm.

Conformational Transitions. pH-dependent conformational transitions were measured by using tryptophan fluorescence in the absence and presence of KCl or sodium sulfate. Ionic strength of the buffer component was kept at 0.05 M. The buffer solutions used were sodium phosphate between pH 2 and 3.5 and between pH 6 and 8, sodium acetate between pH 3.5 and 6, CHES between pH 8 and 10, and CAPS between pH 10.0 and 11.5. The solutions below pH 2 and above pH 11.5 were prepared by using the appropriate concentration of HCl or NaOH, respectively. Measurements were made several hours after the preparation of solution. For the solutions of pH's below 2.5 and above 11.5, the fluorescence signal was monitored as a function of time. The kinetics of denaturation were fast under such conditions, and the reactions were complete within a few minutes. The final value of the fluorescence intensity was stable at acidic conditions but gradually decreased under alkaline conditions due to chemical degradation of the protein; consequently, we used the kinetically determined infinity values for the alkaline pH region to make the transition curve.

The salt-dependent conformational transitions were measured at pH below 2 by fluorescence and at pH above 12 by fluorescence and CD. The protein solutions were prepared by initially bringing the enzyme to the desired pH value at low ionic strength and then increasing the salt concentration. The rates of the salt-dependent transitions were very fast (unpublished results), and the measurements were made soon after the preparation of the solution to minimize the effects of aggregation at low pH and chemical degradation at high pH.

ANS was added to the protein solutions with different concentrations of salt at acidic pH, alkaline pH, or neutral pH to study the interaction with ANS. Because the fluorescence of ANS showed substantial time-dependent increases at acidic pH, the measurements were made at various times after the preparation of solution. At other values of pH the solutions were stable and the measurements were made soon after the preparation of solution.

All measurements were carried out at 20 °C unless otherwise specified, and the temperature was maintained with thermostatically controlled cell holders.

Activity Assay. β-Lactamase concentrations were determined with benzylpenicillin assays. Aliquots (20 μL) of enzyme were added to 2.2 mM benzylpenicillin in 50 mM sodium phosphate buffer (1.0 mL) at pH 7.0 and thermostated at 25 °C, and the linear decrease in absorbance at 240 nm was followed. The concentration of β-lactamase was estimated by using k_{cat} = 2000 s^{-1} and the change in molar extinction coefficient of benzylpenicillin at 240 nm equal to 570 M^{-1} cm^{-1}. The concentration of the enzyme was also determined spectrophotometrically by using a molar absorption coefficient at 280 nm of 27 700. Both measurements agreed well. The reversibility of the acidic and alkaline transitions was measured by using the catalytic activity assay. Aliquots of 20 μL of the enzyme from the sample mixture were assayed as described above, and the activity was compared with that of the native enzyme to determine the percentage recovery.

Light Scattering Measurements. The diffusion coefficient D of β-lactamase was measured with the dynamic light scattering method (Berne & Pecora, 1976). A Brookhaven Instruments Model BI-2030 apparatus was used, with incident beam of λ = 514.5 nm. Details of the instrument and methodology have been reported (Thompson, 1988). The measurements were made at protein concentrations of 90 μM at 20 °C. Three different scattering angles (90°, 60°, and 30°) were used, and no dependence of the diffusion coefficient on the scattering angle was observed. The data were analyzed by the method of cumulants. The polydispersity was in the range of 0.1–0.2 for the native sample and 0.2–0.4 for the other samples. The hydrodynamically equivalent diameter of the protein molecule, a, was calculated from the measured value of D, by using the Einstein–Stokes relation, $a = kT/(3\pi\eta D)$, where k is the Boltzmann constant, T is the temperature in K, and η is the viscosity of the solvent.

Results

Fluorescence Measurements. β-Lactamase I from *B. cereus*

Table I: Reversibility of β-Lactamase I Transitions as a Function of pH and Salt (KCl) Concentration

process	protein concentration[a] (μM)	temperature[a] (°C)	reversibility[b] or $t_{1/2}$ for loss of activity
acidic transitions[c]			
N → U → N (low salt)	20	20	reversible
N → A → N (high salt)	20	20	6 min
	20	5	>12 h
	1	20	5.3 h
	1	5	>24 h
N → A → U → N[d]	20 (A), 1 (U)	20	reversible
alkaline transitions[c]			
N → U → N (low salt)	10	20	~4 h
N → B → N (high salt)	10	20	~9 h

[a] Low-salt conditions were as follows: ionic strength of buffer = 0.05 M. High salt concentrations were as follows: 0.45 M KCl (I = 0.50 M) for the acidic transitions and 1.5 M KCl (I = 1.55 M) for the alkaline transitions. [b] Reversibility was measured by catalytic activity. In those cases where the reversibility was time-dependent the half-life of the loss of reversibility is given. [c] The pH was 7.0 for the N state, 1.7 for the A state, and 12.2 for the B state. [d] The first transition was from N at high salt to A at high salt. The second was to U at low salt, and the final was to N at low salt. The transition from A to U involved a 20-fold dilution.

has three tryptophan residues at positions 210, 229, and 250 (Madgwick & Waley, 1987). The fluorescence spectrum of the native protein exicted at 280 nm had an emission maximum at 337 nm (Figure 1). The fluorescence emission at 337 nm was monitored as a function of pH in the absence and presence of added salt (Figure 2). Cooperative transitions were observed under acidic and alkaline conditions. In the absence of salt (I = 0.05 M), the acidic and alkaline transitions occurred below pH 4 and above pH 10.0, respectively. The midpoint pH of the transitions was 3.0 for the acidic transition and 10.9 for the alkaline transition. As shown in Table I, the acidic transition at low-salt conditions was reversible, while the reversibility of the alkaline transitions depended on the length of time at the high-pH conditions. The half-life for the irreversible inactivation at pH 12.2 was calculated to be 4 h from the time dependence of the amount of reactivation (return of catalytic activity). We attribute this time-dependent lack of reversibility at high pH to irreversible inactivation due to base-catalyzed chemical damage to the protein (Ahern & Klibanov, 1985).

Figure 1 shows the fluorescence spectra of the β-lactamase after the acid and alkaline transitions were complete. In the absence of added salt the emission maximum was 350 nm for both cases, which indicates the full exposure of tryptophan

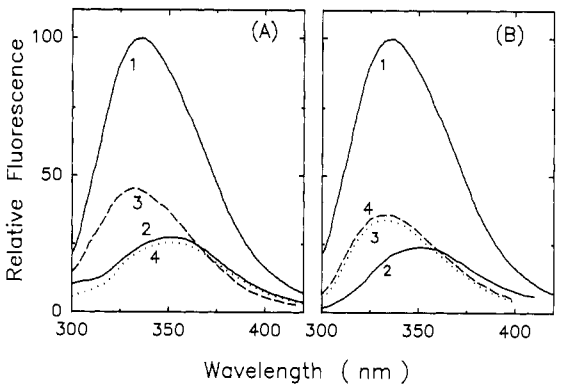

FIGURE 1: Fluorescence emission spectra of β-lactamase at 20 °C. (A) Curves: 1, no salt at pH 7.0 (native); 2, no salt at pH 1.8 (unfolded); 3, in 0.45 M KCl at pH 1.8 (state A); 4, in 4 M Gdn·HCl at pH 7.0. (B) Curves: 1, in 0.45 M KCl at pH 7.0 (native); 2, no salt at pH 12.2 (unfolded); 3, in 1.5 M KCl at pH 12.2 (state B); 4, in 1 M sodium sulfate at pH 12.2 (state B). The ordinate represents the fluorescence relative to that in no salt at pH 7. Excitation was at 280 nm. The protein concentration was 1 μM.

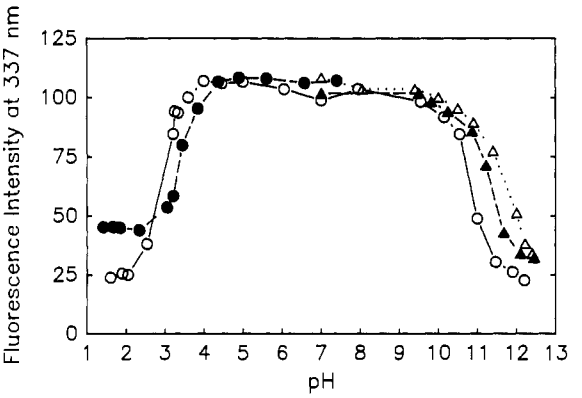

FIGURE 2: pH dependence of the fluorescence emission intensity at 337 nm of β-lactamase at 20 °C: (○) no salt; (●) in 1 M KCl; (▲) in 1.5 M KCl; (△) in 1 M sodium sulfate. Excitation was at 280 nm. The protein concentration was 1 μM.

residues to the solvent; in addition, the tryptophan fluorescence was greatly quenched compared to that of the native state, and the spectra are very similar to that found with 4 M Gdn·HCl (Figure 1A, Table II). In the presence of 1.0 M KCl, the acidic transition began at pH 4.5 and the midpoint pH was 3.4 (Figure 2); thus the transition occurred 0.5 pH unit higher than in the absence of salt; in addition, the final fluorescence intensity at 337 nm was higher than the intensity in the absence of salt (Figure 1). A similar transition was observed in the

Table II: Properties of Different Conformational States of β-Lactamase at 20 °C

		native-like CD spectra			Trp fluorescence spectra			ANS
form	condition	far-UV	near-UV	α-helix content (%)[a]	max wavelength (nm)	rel intensity at max	apparent diameter[b] (Å)	fluorescence at 470 nm[c]
native (N)	pH 7, I = 0.05	+	+	26.4	337	100		0
	pH 7, 0.5 M KCl	+	+	26.3	337	105	45 ± 1	0
	pH 7, 1.5 M Na_2SO_4	+	+	24.3	337	110	47 ± 1[d]	
unfolded by acid (U)	pH 2, I = 0.05	−	−	3.8	350	27	110 ± 2	13 (20)[e]
acidic (A)	pH 2, 0.5 M KCl	+	−	24.6	332	45		150 (600)[e]
unfolded by base (U)	pH 12, I = 0.05	−	−	6.1	350	25	116 ± 1	3
basic (B)	pH 12, 1.5 M KCl	+	−	20.3	332	35	62 ± 1	53
	pH 12, 1.5 M Na_2SO_4	+	−	24.0	332	34		95
unfolded by Gdn·HCl (U)	pH 7, 4 M Gdn·HCl	−	−	0	350	25	(100)[f]	0

[a] Calculated from $f_H = -([\theta]_{222} + 2340)/30300$ (Chen et al., 1972). [b] The hydrodynamically equivalent diameter was calculated from the value of the diffusion constant measured by the dynamic light scattering method using the Stokes–Einstein relationship. The scattering angle was 90°. [c] The fluorescence intensity of the solution containing 1 μM enzyme and 20 μM ANS was measured. [d] For 1.5 M KCl, pH 7.0. [e] The fluorescence intensity increased with time. The values are 10 min and 1 day after preparation of solution. [f] Calculated by the method of Tanford (1968, 1970); see text.

presence of 0.45 M KCl (not shown).

The fluorescence spectra indicate that in the absence of salt the acidic transition leads to the unfolded state (acidic U state), whereas at high concentrations of salt a new intermediate state, state A, is formed with buried tryptophan residues. The emission maximum for state A was 332 nm (Figure 1A, Table II). The intensity at the maximum wavelength was about 65% higher than that of the acid-unfolded conformation in the absence of salt (Figure 1). The reversibility (measured by the return of catalytic activity) of the acidic transition at high-salt conditions decreased greatly with increasing length of time under the acidic conditions. Values for the half-life for loss of reversibility under various conditions are shown in Table I. At 5 °C, the acidic transition was shifted to lower pH by 0.5 pH unit (not shown), and the reversibility improved, the half-life for the loss of reversibility being in excess of 12 h (Table I). As will be shown later, this irreversibility is due to aggregation of the protein under acidic and high-salt conditions. As would be expected, the aggregation increased with increasing protein concentration. The inactivated species at high-salt conditions at acidic pH, however, recovered its activity fully if the salt concentration was first lowered (by dilution) at acidic pH to dissociate the aggregates before the pH was increased to neutral pH (Table I). The fluorescence spectrum of the A state at low pH and high-salt conditions was stable for at least 2 h at the protein concentration used in the fluorescence measurements (1 μM). This indicates that the small amount of aggregation which occurs during this time period does not affect the fluorescence properties.

As shown in Figure 2, the alkaline transition is also dependent on the presence of salt. The presence of 1.5 M KCl shifted the transition by 0.5 pH unit to a higher pH region, and the fluorescence spectrum after the transition showed an emission maximum at 332 nm and a higher intensity than the spectrum in the absence of salt (Figure 1B, Table II). A similar spectrum was obtained in the presence of 1.0 M sodium sulfate at pH 12.2, where the alkaline transition was assumed to be almost over. The effect of sodium sulfate on shifting the alkaline transition to the higher pH region was greater than that of KCl; that is, an equivalent effect on the transition was observed with a lower concentration of sodium sulfate. The reversibility of the alkaline transition in the presence of 1.5 M KCl was much better than that in the absence of salt, and the half-life for the loss of reversibility at pH 12.2 was more than 8 h, compared to about 4 h in the absence of salt (Table I). Thus an analogous situation exists at high pH in that the presence of high concentrations of salt induces a unique conformational state (state B), while the low ionic strength conditions (I = 0.05 M) lead to the unfolded state (the alkaline U state).

Figure 3 shows the cooperative nature of the transitions from the acidic U state to the A state and the alkaline U state to the B state as a function of salt concentration and measured by the change in tryptophan fluorescence. The transition from the acidic U state to the A state at pH 1.6 induced by KCl was highly cooperative and had a midpoint at 0.12 M KCl. The transition from the alkaline U state to the B state at pH 12.2 induced by KCl was less cooperative than the acidic transition but still showed saturation at high salt concentrations. The midpoint (0.6 M KCl) was higher than that of the acidic transition. Sodium sulfate induced a similar, but more cooperative, transition with the midpoint (0.3 M) about half of that for the transition induced by KCl. The spectra at different concentrations of salt gave isoemissive points for the respective transitions, indicating a two-state transition between

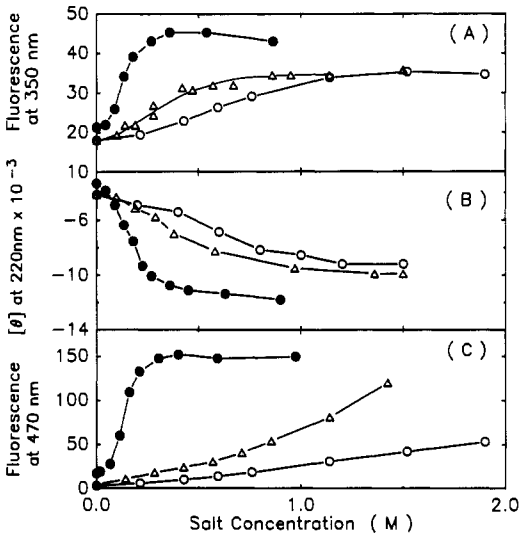

FIGURE 3: Dependence on the salt concentration at 20 °C of the tryptophan fluorescence intensity at 330 nm (A), the ellipticity at 220 nm of β-lactamase (B), and the ANS binding to β-lactamase measured by the fluorescence at 470 nm (C). (●) KCl concentration dependence at pH 1.6; (○) KCl concentration dependence at pH 12.2; (△) sodium sulfate concentration dependence at pH 12.2. (A) Excitation was at 280 nm. The ordinate shows the fluorescence intensity relative to the maximal intensity of the native protein at pH 7.0. The protein concentration was 1 μM. (B) The protein concentration was 5 μM. (C) The β-lactamase concentration was 1 μM; that of ANS was 20 μM. The measurements were performed 10 min after preparation of the solution. Excitation was at 400 nm.

the U state and A or B states.

Circular Dichroism Spectra. The CD spectra of native β-lactamase at pH 7.0 are shown in Figure 4A for different salt concentrations and for the protein unfolded in 4 M Gdn·HCl. In the absence of salt, the CD spectrum in the far-UV region for the native protein shows minima at 220 and 208 nm and a maximum at 192 nm. Analysis of the CD spectrum for the native protein by the method of Chen et al. (1972) indicates that the content of α-helix is about 26% (Table II). In the near-UV region, the spectrum shows a broad negative peak with a minimum at 275 nm. The addition of KCl or sodium sulfate up to 1.5 M did not change the CD spectrum significantly. In the presence of 4 M Gdn·HCl, the β-lactamase unfolded completely and the CD spectrum showed loss of intensity above 220 nm and a sharp decrease below 210 nm. The near-UV region showed negligible ellipticity.

The CD spectra at pH 1.8 are shown in Figure 4B in the presence and absence of 0.45 M KCl. In the absence of KCl, the CD spectrum in the far-UV region was very different from that of the native protein at pH 7.0. It showed a large negative trough at 198 nm, characteristic of unfolded protein. The spectrum is similar to, although not identical with, the spectrum for the protein unfolded by 4 M Gdn·HCl at pH 7.0. In the presence of 0.45 M KCl, which is sufficient concentration to cause all the protein to be in the A state (Figure 3), the spectrum was very similar to that of the native protein. The ellipticity at 208 nm was 11 000 for state A, compared to 12 000 for the native state at pH 7.0 in the absence of salt. The content of α-helix in the A state (25%) is similar to that in the native protein (26%) (Table II). On the other hand, the CD spectrum in the near-UV region was independent of salt and closely resembled that of the unfolded protein. As will be described later, the A state aggregated with time. To determine if the aggregation affected the CD spectrum, we measured the CD spectrum of the A state at different times after preparation of the solution at 20 or 5 °C. The effect of

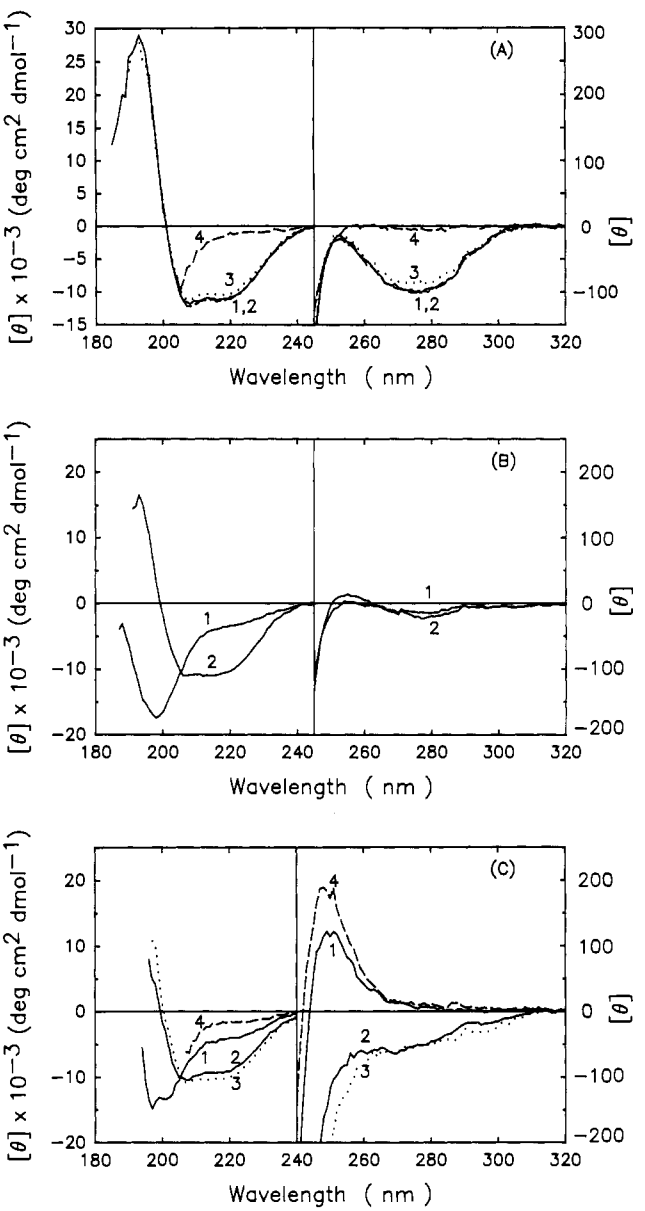

FIGURE 4: Circular dichroism spectra of β-lactamase at pH 7.0 (A), at pH 1.8 (B), and at pH 12.0 (C) at 20 °C. (A) Curves: 1, no salt; 2, in 1.5 M KCl; 3, in 1.5 M sodium sulfate; 4, in 4 M Gdn·HCl. (B) Curves: 1, no salt; 2, in 0.5 M KCl. (C) Curves: 1, no salt; 2, in 1.5 M KCl; 3, in 1.5 M sodium sulfate; 4, in 4 M Gdn·HCl. The protein concentration was 20 μM.

aggregation on the CD spectrum was negligible for at least several hours after the formation of the A state.

The CD spectra at pH 12.0 are shown in Figure 4C under various salt conditions. Without salt, the CD spectrum in the far-UV region was similar to that for the protein in the absence of salt at pH 1.8 and to the spectrum in 4 M Gdn·HCl at pH 12. The spectrum showed a large positive peak at 245 nm. A similar peak was observed for the spectrum in 4 M Gdn·HCl at pH 12. This peak arises from the ionized form of the tyrosine residues; the pK_a for solvent-exposed tyrosine is about 10 (Tanford, 1970). Other than this sharp peak at 245 nm, the spectrum without salt was featureless in the near-UV region. This shows that the protein was substantially unfolded at pH 12 under low-salt conditions. In the presence of 1.5 M KCl or 1.5 M sodium sulfate, which were high enough concentrations to induce the B state (Figure 3), the spectrum in the far-UV region was similar to that of the native state for both salts. The ellipticities at 208 nm in 1.5 M KCl and 1.5 M sodium sulfate were 10 400 and 10 800, respectively, and these correspond to a helix content of 20% and 24%, respectively (Table II). In the near-UV region the spectrum in 1.5 M KCl or 1.5 M sodium sulfate showed a gradual decrease below 300 nm and a sharp decrease below about 250 nm. There was no peak corresponding to the tyrosine ionization. The negative intensities at around 270 nm were smaller than those of the native state.

The transitions from the acidic U state to state A, and from the alkaline U state to state B, as a function of salt concentration, were measured by the ellipticity change at 220 nm (Figure 3B). The transitions were cooperative and similar to those observed by tryptophan fluorescence; the midpoint was 0.15 M for the KCl-induced transition at pH 1.6, 0.58 M for the KCl-induced transition at pH 12.2, and 0.38 M for the sodium sulfate induced transition at pH 12.2. These values are consistent with the values measured by tryptophan fluorescence.

Light Scattering Measurements. Table II shows the apparent hydrodynamic diameter of the β-lactamase determined by dynamic light scattering under different conditions of pH and salt. The overall dimensions of the native protein from the X-ray crystallographic study (Samraoui et al., 1986) are approximately 33 Å × 38 Å × 49 Å. The apparent diameter at pH 7.0 in 0.5 M KCl was 45 Å, which is consistent with the crystallographic data. At pH 12, the apparent diameter was 116 Å under low-salt conditions, corresponding to a 17-fold increase in volume over that of the native state. At pH 12.5 and 1.5 M KCl, the B state had an apparent diameter of 62 Å, a 2.6-fold increase in volume over that of the native state. We can estimate the apparent hydrodynamic diameter for the fully unfolded state using the equations proposed by Tanford (Tanford et al., 1967; Reynolds & Tanford, 1970):

$$[\eta] = (2.5N/M_r)(4/3\pi R_s^3) = 0.72n^{0.66}$$

where $[\eta]$ is the intrinsic viscosity, R_s is the Stokes radius, N is Avogadro's number, M_r is the molecular weight, and n is the number of residues. The calculated apparent diameter for the fully unfolded state is 100 Å, corresponding to an 11-fold increase in volume over that of the native state. These results show that the compactness of the B state is somewhat expanded compared to that of the native state, but much more compact than the unfolded state. There was no evidence of any time-dependent change in the hydrodynamic radius over a period of time during the light scattering measurements for the B, N, and acidic and alkaline U states, consistent with the absence of aggregation even at the high protein concentration used (90 μM).

Measurement by dynamic light scattering of state A showed a large increase in apparent size with time reflecting time-dependent aggregation of the A state at the high protein concentrations used in the experiment. As noted, the acidic U state did not exhibit time-dependent aggregation and gave an apparent diameter of 110 Å. As was the case for the alkaline unfolded state, this value is larger than expected for the monomeric unfolded state and is consistent with previous reports that proteins may be more expanded due to charge repulsion in the pH-unfolded states relative to that at neutral pH in Gdn·HCl (Tanford, 1968; Babul & Stellwagen, 1972).

To examine the nature of the aggregation at acidic and high-salt conditions, we followed the time-dependent change in Rayleigh scattering intensity at 330 nm of the A state. The amount and rate of aggregation were very dependent on the protein concentration and temperature; it was slower at lower protein concentrations and lower temperature. The results show that the protein concentration must be less than 8 μM at 5 °C to prevent aggregation of the A state.

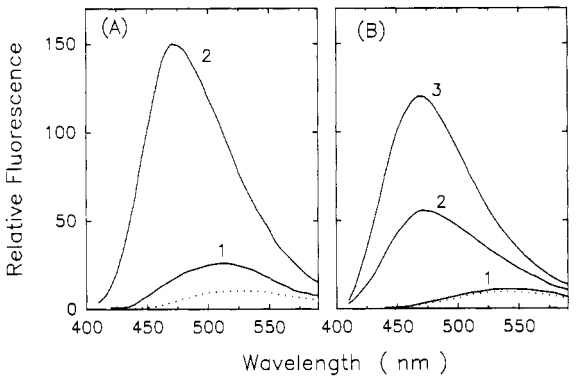

FIGURE 5: Fluorescence spectra of 20 μM ANS in the presence of 1 μM β-lactamase at pH 1.7 (A) or pH 12.2 (B) at 20 °C. (A) Curves: 1, no salt; 2, in 0.6 M KCl. (B) Curves: 1, no salt; 2, in 1.5 M KCl; 3, in 1.5 M sodium sulfate. Dotted lines show the fluorescence of 20 μM ANS in the absence of protein. The measurements were performed 10 min after preparation of the solution. Excitation was at 400 nm.

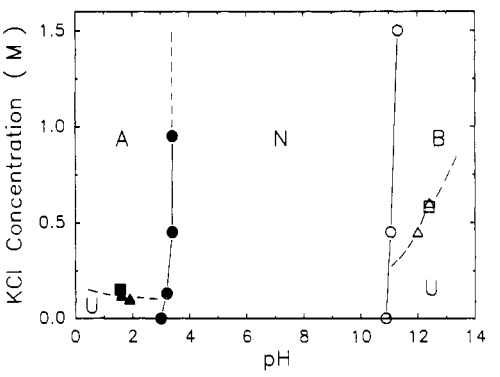

FIGURE 6: Phase diagram for the KCl-dependent conformational states of β-lactamase at 20 °C: (N) native state; (A) acidic molten-globule state; (B) basic molten-globule state; (U) unfolded state. The phase boundary corresponds to the midpoint of the conformational transitions. The squares at pH 1.6 and 12.2 were determined from the change in CD at 220 nm; the other points were determined from the change in tryptophan fluorescence.

ANS Binding. The fluorescence emission of ANS is known to increase when the dye binds to hydrophobic regions of a protein (Stryer, 1965). The fluorescence of ANS increased significantly in the presence of the A or B state of β-lactamase. The change in the fluorescence of ANS in the presence of the native protein at pH 7.0 was negligible and was independent of the salt concentration. Figure 5A shows the fluorescence spectra of ANS in the presence of the acidic U or A state. While a small increase in fluorescence was observed in the presence of the U state, a very large increase was observed in the presence of the A state. The emission maximum shifted from 540 to 470 nm, indicating the burial of the ANS molecule in a hydrophobic environment (Turner & Brand, 1968). After an initial rapid increase that occurred in a few minutes, the fluorescence increased slowly with time. Figure 3C shows the dependence of ANS fluorescence intensity as a function of KCl concentration. The transition was cooperative with a midpoint at 0.11 M KCl, which is very similar to the midpoint for the U to A state transition as measured by tryptophan fluorescence (Figure 3A) and ellipticity at 220 nm (Figure 3B). A detailed analysis of the ANS binding to the A state was not possible because the reaction was complicated by the binding of ANS to both the monomeric and aggregated A states.

Figure 5B shows the fluorescence spectra of ANS in the presence of the alkaline U state or state B. There was no increase in ANS fluorescence in the presence of the alkaline U state. A significant increase in fluorescence was observed in the presence of the B state formed by either sodium sulfate or KCl. The fluorescence of ANS in the presence of the B state did not show a time-dependent change, consistent with the absence of aggregation in the B state. The emission intensity increased with the concentration of salt but did not show a cooperative transition as was observed for the acidic transition (Figure 3C). The fluorescence intensity in the presence of sodium sulfate was higher than that in the presence of KCl; however, the increase in intensity of the ANS fluorescence in the presence of the B state was smaller than that in presence of the A state.

Since the ANS fluorescence in the presence of state B was stable, we determined the stoichiometry of the ANS binding by fluorescence titration of the B state with ANS. When 40 μM state B (in 1.5 M KCl) was titrated with ANS, the increase in fluorescence was proportional to the ANS concentration below 20 μM ANS. From this linear portion, we calculated that the maximal increase in fluorescence corresponds to the binding of 1 mol of ANS/mol of protein. In the presence of 100 μM ANS about 2 mol of ANS bound to the B state. A Scatchard plot for ANS binding was not linear, indicating multiple binding with different affinities; a dissociation constant of 25 μM was estimated for the first two ANS molecules.

Discussion

Phase Diagram of β-Lactamase Conformational States. A particularly significant finding from this investigation was that the conformation of β-lactamase depends not only on pH but also on the nature and concentration of salt. Figure 6 shows a phase diagram of the conformational states of β-lactamase as a function of pH and KCl. The boundaries of the phases correspond to the midpoints of the measured conformational transitions. There are five conformational states: native, acidic U, A, alkaline U, and B.

The acidic and alkaline U states, which are present under conditions of low ionic strength at the extremes of pH, have many similar properties: (1) Their tryptophan fluorescence emission spectra have maxima at 350 nm, indicating complete exposure of the tryptophan residues to the solvent. (2) Their far-UV CD spectra show the loss of ordered secondary structure. The spectra are similar to, but not identical with, to the spectra in 4 M Gdn·HCl. (3) Their near-UV CD spectra show no fine structure except the positive peak at 250 nm for the B state which is due to the ionized tyrosine residues. These results indicate that the protein is almost completely unfolded under these conditions.

For the same conditions of pH, but at higher salt conditions, β-lactamase I assumes conformational properties, states A and B, consistent with those of the molten-globule state. The A and B states also have many properties in common. These include the following: (1) The far-UV CD spectra show that the secondary structure content of states A and B is similar to that of the native state, although there is a small decrease in the helical content. (2) The tryptophan fluorescence spectra are similar, with a maximum at 332 nm, blue shifted from that of the native state (λ_{max} = 337 nm), indicating an environment apparently more hydrophobic than that of the native state for the tryptophan side chains. The fluorescence emission of states A and B is substantially quenched compared to that of the native protein. This suggests that the side chains of both A and B states are more mobile, resulting in dynamic quenching of the exicted state. The B state shows no peak at 250 nm in the near-UV CD corresponding to the ionized tyrosine residues. This suggests that the environment of the tyrosine residues in state B is hydrophobic, since the pK_a would be

increased in a hydrophobic environment. (3) Both A and B states bind ANS strongly, whereas the hydrophobic dye does not bind to either the native or unfolded protein. This indicates the presence of solvent-accessible hydrophobic regions, of either dynamic or static nature, in the A and B states. ANS binds more tightly to state A than to state B; since ANS has a negative charge, due to the sulfonyl group, over the pH range of 1.5–12 (Turner & Brand, 1968), this may contribute to the tighter binding to state A, which would be positively charged, in contrast to state B, which would be negatively charged (see below). Very recently, Semisotnov et al. (1987) have reported that ANS binds to a transient intermediate during the refolding of carbonic anhydrase. This intermediate exhibits properties expected of a molten globule; analogous to the situation with β-lactamase ANS does not bind to native or unfolded carbonic anhydrase. Since ANS is well-known to bind to hydrophobic regions, its binding may be a characteristic of molten-globule-like states.

In spite of the similarities between the A and B states, there are also definite differences. These include the following: (1) Whereas state A shows a very weak and featureless CD spectrum in the near-UV region, indicating the essential absence of the native-like tertiary structure, that of state B has a distinct negative intensity below 300 nm. This suggests that state B still retains some ordered tertiary structure. (2) The salt-dependent transition from the acidic U state to the A state is more cooperative than the transition from the alkaline U state to the B state. The midpoint salt concentration for the latter is higher than that of the former; that is, the U to B transition is less sensitive to the ionic conditions than the U to A transition. (3) State A shows a strong tendency to aggregate whereas B state does not aggregate. The absolute value of the net charge of β-lactamase I, estimated from the amino acid composition, is very similar at either extreme of pH, namely, +36 to +39 at pH 2, depending on the signal peptide processing site at the amino-terminal end, and −32 at pH 12. Therefore, the strong tendency for aggregation of state A is not readily explained by a difference in the net charge, and in fact, the acidic U state does not aggregate in the same pH region. The temperature-dependent aggregation of the A state suggests that more hydrophobic surface is accessible to the solvent than in state B, thus facilitating intermolecular interactions. The stronger ANS binding to state A may also reflect a more accessible hydrophobic region of state A compared to state B, although it is difficult to separate this effect from that due to the negative charge of ANS described above.

Mechanism of the Salt-Dependent Conformational Transitions. Under conditions of extreme pH's, the main forces to unfold the protein are the repulsions between charged groups on the protein molecule. The presence of high concentrations of salts will have two effects on the protein conformation. The presence of counterions around the charged groups will weaken these repulsions and thus permit other forces favoring folding to become relatively strengthened and thus manifest themselves. In addition to this nonspecific effect of salts as counterions, specific effects of salts on protein folding will come into play. The stabilizing or destabilizing effects of salts on proteins arise either by effects on water structure, and hence hydrophobic interactions, or by interactions with charged groups. The net effect on the stability of the protein is determined by the relative effects on the free energies of both the unfolded and native states. The stabilizing effect of salts on proteins follows the Hofmeister series [e.g., von Hippel and Wong (1965)]. Sodium sulfate is much more effective than KCl in stabilizing hydrophobic interactions and in increasing the stability of proteins. Arakawa and Timasheff (1982) have shown that preferential hydration of proteins occurs in the presence of salts such as NaCl, sodium acetate, and sodium sulfate and that the resulting unfavorable free energy of the unfolded state is related to the stabilizing effect of these salts on proteins. The relative effectiveness of KCl and sodium sulfate in bringing about the U to A or B transitions is consistent with their chaotropic effects and hence the involvement of hydrophobic interactions.

The U to A transition is highly cooperative at low concentrations of KCl, while the U to B transition is less cooperative and the midpoint concentration of the salt is much higher than for the acid transition. These differences may reflect relative differences in the contribution of the two factors; the specific effects of salt may be more important in the alkaline U to B transition. In any case, it is evident that, in both the A and B states, hydrophobic interactions become stronger compared to the U state. The buried tryptophan residues in the A and B states, and the buried tyrosine residues in the B state, indicate the presence of a significant nonpolar core and show the importance of hydrophobic interactions in the formation of the A- and B-state conformations. The binding of ANS to the A and B states and the temperature-dependent aggregation of the A state also indicate the presence of exposed hydrophobic surface. The loss of these properties on going to the native state indicates that additional hydrophobic interactions are involved in the transition between the molten-globule conformation and the native protein. The high net charge and the effective increased size of the charged residues due to the presence of the associated counterions on the protein molecule may prevent the formation of native tertiary structure in the extreme-pH regions and thus stabilize the intermediate conformational states.

It is well established that hydrophobic interactions are the main driving force for protein folding and hydrogen bonding and electrostatic interactions contribute to guiding the folding pathway [reviewed in Jaenicke (1987)]. The stability of the A- and B-state conformations, and the rapidity of their formation from state U (unpublished observations), is consistent with the results involving the molten-globule state in other proteins (Dolgikh et al., 1984, 1985; Kuwajima et al., 1985; Ikeguchi et al., 1985, Semisotnov et al., 1987) and suggests that such states might be the normal unfolded form of the protein (rather than the random coil state) under many conditions (e.g., high temperature).

Related Conformational States in Other Proteins. β-Lactamase from *S. aureus* has been shown to have an intermediate state, state H, which is predominant in the transition region at low concentrations of urea and Gdn·HCl (Robson & Pain, 1976a,b; Carrey & Pain, 1978; Mitchinson & Pain, 1985). This intermediate state retains much of the native secondary structure but lacks the tertiary structure. The equivalent hydrodynamic volume of state H is eight times that of the native state (Carrey & Pain, 1978) in contrast to the 2.6-fold expansion of state B with the *B. cereus* enzyme. An analogous intermediate to state H was not observed in the urea and Gdn·HCl-induced unfolding of β-lactamase I from *B. cereus* (A. L. Fink and L. J. Calciano, unpublished results). At very low urea concentrations a more compact conformational state was observed as a transient intermedite in refolding of the staphylococcal β-lactamase when monitored in urea-gradient electrophoresis (Creighton & Pain, 1980). Two mutants of this enzyme appear to have their folding blocked at a step prior to the native state and to adopt compact conformations closely resembling the native state, with native-like

secondary structure but reduced aromatic circular dichroism (Craig et al., 1985). An additional conformational state, A, has been observed for the enzyme from *S. aureus* at low pH, although its salt dependence has not been reported (Adams et al., 1980). The CD spectrum of this state at pH 2.6 [Figure 6 of Adams et al. (1980)] corresponds closely to that for a 1:1 mixture of the A state and acidic U state for β-lactamase from *B. cereus*, suggesting the existence of a similar salt-dependent conformational transition.

Salt-dependent conformational transitions at acidic pH's from a largely unfolded state to an intermediate conformational state have been reported for cytochrome *c* (Stellwagen & Babul, 1975; Ohgushi & Wada, 1983) and for human interferon γ (Arakawa et al., 1987). These results suggest that the salt-dependent conformational transitions at acidic or alkaline pH regions may be a general property of many proteins. The midpoint salt concentration of the transition, however, will depend on the particular protein and other conditions, as is the case for the difference between the U to A transition and the U to B transition with β-lactamase. If the stability of the intermediate conformation is high enough, the fully unfolded conformation will not be observed, even at very low salt conditions, consistent with reports for residual structure for several proteins.

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