Side-Chain Mobility of the β-Lactamase A State Probed by Electron Spin Resonance Spectroscopy†

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ABSTRACT: β-Lactamase from *Bacillus licheniformis* forms a stable compact intermediate state at low pH and moderate salt concentration (the A state), with properties consistent with a molten globule. A single cysteine residue was introduced into this class A β-lactamase by site-directed mutagenesis at position 166. A spin label was attached to the thiol of this cysteine residue via a disulfide bond as a probe of the side-chain mobility. The mutant protein and the spin-labeled derivative exhibited similar conformational properties to the wild-type enzyme at acidic pH. The A state induced by chloride or trichloroacetate (TCA) anions was characterized by circular dichroism and esr. The A state at pH 0.5 (0.32 M HCl), or at pH 2 in the presence of 8 mM TCA or 0.4 M Cl^-, had comparable amounts of secondary structure to the native state but lacked significant tertiary structure, as judged by the lack of near-UV circular dichroism. Analysis of the esr spectral line widths showed that the mobility of the spin label in the A state was similar to that in the native state and much less mobile than in the unfolded state, indicating significant constraints on the side-chain mobility in this region of the molecule in the A state. The implications of this finding to the structure of the A state are discussed.

Intermediate states of proteins have now been observed for many proteins under both equilibrium and kinetic conditions (Ptitsyn, 1987; Kuwajima, 1989; Goto et al., 1990a; Christensen & Pain, 1991). In some cases these are quite compact and have been called molten globules (Ohgushi & Wada, 1983). Characteristic features of these partially folded states include the presence of significant secondary structure, little nativelike tertiary structure, and substantial exposed hydrophobic surface area (Baldwin, 1991). Relatively little is known about the detailed structures of such intermediates, with the exception that at least some of the secondary structure present is also present in the native state (Dolgikh et al., 1985; Damaschun et al., 1986; Baum et al., 1989; Hughson et al., 1990; Jeng & Englander, 1991).

The apparent lack of significant tertiary structure in compact intermediate states suggests that there is minimal interaction between side chains, in contrast to the native state where side chains are normally tightly packed and interdigitated. One means of probing the mobility of side chains is to replace them with a spin label and use electron spin resonance spectroscopy (esr) to measure the mobility of the label. This method has been pioneered by Hubbel and co-workers (Todd et al., 1989), who have used it to determine structural characteristics of colicin E1. The basis of this approach is to attach a spin label to a unique Cys residue introduced by site-directed mutagenesis. Measurements of the esr spectrum of the spin label can then be used to determine its local environment, including its mobility, from the shape of the spectral signal. Analysis of the line shape provides information about the motional amplitude and frequency of the nitroxide and the local solvent polarity (Todd et al., 1989). The mobility of the nitroxide, as determined from the esr spectrum, reflects both the motion of the spin label relative to the protein and that of the protein itself.

We have previously shown that β-lactamase from *Bacillus cereus* forms a stable compact intermediate with molten globule-like properties under conditions of low pH and low to moderate anion concentration (Goto et al., 1990a). The homologous β-lactamase from *Bacillus licheniformis* was chosen for the present study because a high-resolution crystallographic structure is available (Moews et al., 1990) and because we have previously generated site-specific mutants using the cloned gene (Ellerby et al., 1990; Escobar et al., 1991). In this report we show that the class A β-lactamase from *B. licheniformis* behaves very similarly at low pH to that from *B. cereus*, even though the isoelectric points are quite different (4.9 and 8.4, respectively). Replacement of Glu-166 by Cys (E166C) has negligible effect on the low-pH conformational properties of β-lactamase; the absence of Cys in the wild-type enzyme thus allowed us to introduce a single spin label at position 166 in the mutant protein. The presence of the spin label also had little effect on the low pH conformational behavior of the protein.

EXPERIMENTAL PROCEDURES

Materials. The cysteine-166 mutant of *B. licheniformis* β-lactamase was constructed and expressed in *Bacillus subtilis* as previously described (Escobar et al., 1991). Methanethiosulfonate spin label [(1-oxyl-2,2,5,5-tetramethyl-3-pyrroline-3-methyl)methanethiosulfonate) (MTSSL) (Berliner et al., 1982)] was obtained from Reanal (Budapest, Hungary). Hydroxy-TEMPO was obtained from Aldrich. Trichloroacetic acid was obtained from Fisher Scientific, ultrapure guanidine hydrochloride (Gdn·HCl) from ICN Biochemicals, and ultrapure urea from Boehringer Mannheim Biochemicals. The concentration of stock solutions of urea was determined by measuring the refractive index according to Nozaki (1972). All solutions used for circular dichroism (CD) and high-performance liquid chromatography (HPLC) were made with HPLC-grade water from Fisher Scientific and filtered through a 0.22-μm Millipore filter. All pH measurements were made with a Beckman F71 pH meter using a microcombination

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glass electrode (Microelectrodes Inc., Model MI-410). Adjustments to solution pH were made using concentrated hydrochloric acid from Fisher Scientific.

Purification of WT and E166C β-Lactamase. Wild-type and mutant (E166C) β-lactamase enzymes were expressed and purified from *B. subtilis* as previously described (Ellerby et al., 1990; Escobar et al., 1991). After the final column fractions containing β-lactamase activity were pooled and exchanged into 0.05 M potassium phosphate, 0.05 M KCl buffer, pH 7.0, by ultrafiltration. Proteins were purified to homogeneity as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the Phastsystem (Pharmacia). The concentrations of protein stock solutions were determined at 280 nm, utilizing a molar extinction coefficient of $2.343 \times 10^4\ M^{-1}\ cm^{-1}$, with a Hewlett-Packard 8452A diode array spectrophotometer.

Protein Spin Labeling. A 5% excess of MTSSL stock solution (20 mM in acetonitrile) was added to a solution of partially unfolded purified protein (30–40 μM in 1 M urea, 0.05 M potassium phosphate, and 0.5 M KCl, pH 7) and allowed to equilibrate (with stirring) in an ice bath for 1 h. Excess label was then removed by washing five times in a Centricon-10 filter at 5000*g* for 30 min. The stoichiometry of the label to enzyme was determined by estimating the spin label concentration from the esr signal intensity, using the corresponding signal from the MTSSL as a standard, and the enzyme concentration from the absorbance at 280 nm (where the spin label has no absorbance). The stoichiometry was found to be 1:1 on a molar basis.

Circular Dichroism. CD spectra were recorded on an Aviv 60DS circular dichroism spectrometer calibrated with (+)-10-camphorsulfonic acid. Far-UV CD measurements were made from 260 to 200 nm using a 1-mm rectangular quartz cell. A 10-mm path length round cell was used to make near-UV (360–260 nm) measurements. Protein concentrations were 15 μM for the CD measurements. The cell compartment was maintained at 25 °C using a Neslab RTE-10 water bath, and data were collected at 1-nm intervals with an averaging time of 5 s unless otherwise specified. Data for thermal denaturation were collected over the 30–90 °C range. A linear temperature programmer (Neslab) was used with a heating rate of 0.5 °C/min. Temperature was monitored via a thermocouple taped to the cell. Spectral changes were monitored continuously at 222 nm. After the protein was denatured, the temperature was lowered quickly (within 15 min) to 25 °C and the reversibility of the transition was determined by recovery of the CD signal. Scans were recorded before and after the thermal denaturation.

HPLC. Size-exclusion chromatography was performed using a Bio-Sil 250 Column (Bio-Rad) (7.5 × 600 mm) on a Beckman HPLC system. Separations were monitored at 215 and 280 nm. For a given set of buffer conditions the column was equilibrated with a minimum of 3 column volumes at a flow rate of 1 mL/min. All solutions were prepared using Fisher Scientific HPLC-grade water, passed through a 0.22-μm Millipore filter, and degassed. Sample volumes were 20 μL of 15 μM protein.

Electron Spin Resonance. Electron spin resonance spectra were measured on a Bruker Model ESP 380 spectrometer in continuous wave mode using a Bruker dielectric resonator with a variable temperature accessory. The dielectric resonator has a high filling factor which increases the esr signal to noise ratio by approximately a factor of 5. This increased sensitivity was essential for producing high-quality spectra from the 15 μM protein samples. The protein samples were 20 μL of 15 μM protein in a 100-μL sealed glass capillary micropipette. The modulation amplitude was 2.0 G for the native and A state samples and 0.5 G for the unfolded samples, and the modulation frequency was 100 kHz for a 60-G scan width.

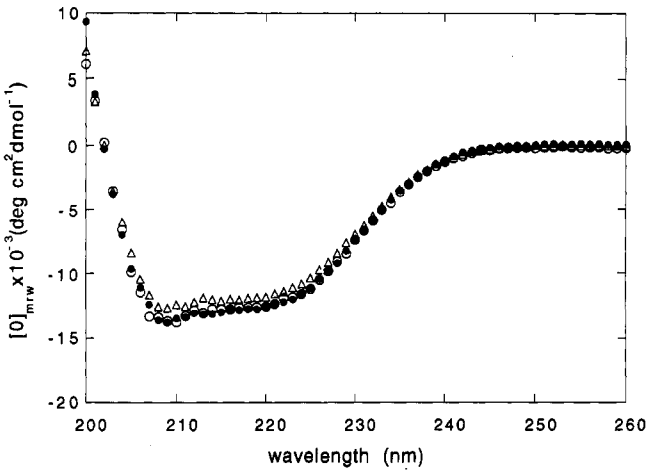

FIGURE 1: Comparison of the far-UV circular dichroism spectra of wild-type β-lactamase (●), the E166C mutant (○), and the spin-labeled derivative of E166C (△). Conditions were pH 7.0, 25 °C, and 15 μM protein.

Calculation of Rotational Correlation Time τ_R from Esr. Esr spectra for $\tau_R > 1$ ns were simulated using the Fortran program developed by Schneider and Freed (1989) adapted for Unix running on a Sun Sparc Server, under SunOS 4.1. The truncation parameters used for the simulations were $L^e_{max} = 14$, $L^0_{max} = 13$, $K_{max} = 10$, $M_{max} = 2$, and $p^l = 2$. The number of Lanczos steps was 128. The spectral parameters for MTSSL were $g_{xx} = 2.0086$, $g_{yy} = 2.0086$, $g_{zz} = 2.0032$, $A_{xx} = 6.23$ G, $A_{yy} = 6.23$ G, and $A_{zz} = 35.7$ G.

Esr spectra for $\tau_R < 1$ ns were simulated using motional narrowing theory, recently applied to spin-labeled peptides (Todd & Millhauser, 1991). The first derivative peak-to-peak line widths and line heights were used to calculate the rotational correlation time τ_R. Care was taken to ensure that these rotational times in the range of 0.5–1 ns were consistent with the method described above by Schneider and Freed.

Viscosities. Temperature-dependent viscosities of solutions used for esr measurements were measured using a Cannon-Ubbelohde semi-microviscometer immersed in a constant-temperature circulating water bath (Neslab).

RESULTS AND DISCUSSION

Comparison of the Structures of E166C and E166C-SL with Those of Wild Type. The far-UV circular dichroism (CD) spectrum of the native E166C mutant of β-lactamase from *B. licheniformis* is compared to that of the wild type in Figure 1. The spectra are superimposable within experimental error, indicating similar secondary structures. Similarly, the CD of the spin-labeled derivative of E166C is essentially the same as that of the wild-type protein (Figure 1). The tryptophan fluorescence emission maximum is the same for the spin-labeled derivative as for the wild-type protein, further indication of a lack of significant structural perturbation of the mutation and labeling.

A measure of the relative stability of the different proteins can be obtained from determination of the position of their thermal denaturation transitions. At pH 7.0 the replacement of Glu by Cys at position 166 caused a minor decrease in the T_m (63.0 ± 0.5 °C for E166C compared to 65.0 ± 0.5 °C for the wild type). On the other hand, the spin-labeled derivative showed a broader (less cooperative) transition, as measured

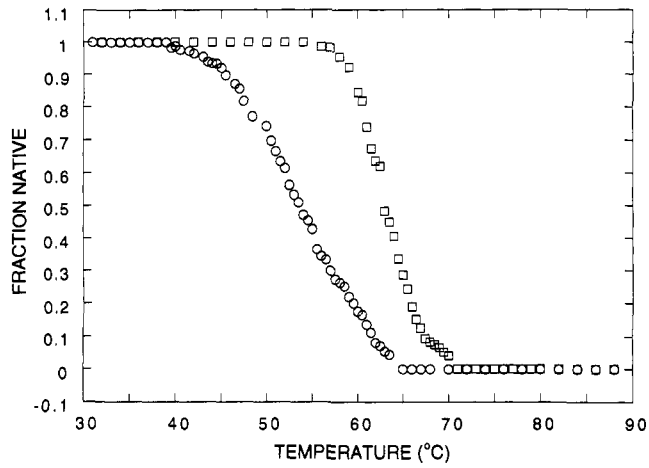

FIGURE 2: Thermal denaturation transitions of E166C β-lactamase (□) and its spin-labeled derivative (○). The transition was monitored by ellipticity at 222 nm. Protein concentration was 15 μM, pH = 7.0.

by ellipticity at 222 nm, beginning around 40 °C, with a midpoint of 53 °C, indicating decreased thermal stability due to the presence of the spin label (Figure 2).

The size of the spin label is similar to that of the tryptophan side chain and somewhat larger than the glutamate side chain. Given that Glu-166 is substantially buried (Knox & Moews, 1991), it is likely that some perturbation of the local structure in the vicinity of the Glu-166 side chain occurs. Examination of the crystallographic structure indicates that this is likely to be quite localized and accommodated by displacement of bound water molecules. Thus the presence of the spin label in the active-site region is anticipated to be accommodated with relatively minor rearrangements.

The catalytic activity of the E166C mutant is dramatically decreased, by 10^6 relative to the wild-type enzyme (W. Escobar, A. Tan, and A. Fink, unpublished observations). This reflects the essential nature of this residue for catalysis.

HCl Titrations of Wild-Type, E166C, and E166C-SL β-Lactamases. As observed with β-lactamase from *B. cereus* (Goto & Fink, 1989; Goto et al., 1990a), the titration of a salt-free sample of wild-type β-lactamase from *B. licheniformis* from neutral pH to pH < 1 leads to an initial unfolding transition in the vicinity of pH 3, yielding the acid-unfolded state (whose far-UV CD spectrum, Figure 3A, indicates a small amount of residual secondary structure), following by a *refolding* transition at pH < 2, culminating in the return of a comparable amount of secondary structure as in the native state by pH 0.5 (Figure 3A). The titration curve for wild-type *B. licheniformis* β-lactamase, as monitored by ellipticity at 222 nm, is shown in Figure 4. Given the different isoelectric points for the enzymes from *B. cereus* (8.6) and *B. licheniformis* (4.9), it is interesting to note that the position of the acid-induced unfolding transition occurs over very similar ranges of pH, the midpoints of these transitions being 2.9 and 2.5, respectively.

Replacement of Glu-166 by Cys had no significant effect on the HCl titration behavior: the protein is unfolded to the same extent in the vicinity of pH 2 and refolds to almost nativelike secondary structure at pH 0.5 (Figures 3 and 4). The CD spectra of E166C as a function of pH are essentially the same as those observed with the wild type (Figure 3B). Similar behavior is seen with the spin-labeled derivative of Cys-166, with the exception that the unfolding transition occurs at slightly higher pH (midpoint pH 3.3) (Figure 4). Thus coupling the spin label to Cys-166 results in some destabilization of the protein, as manifested by the decreased thermal

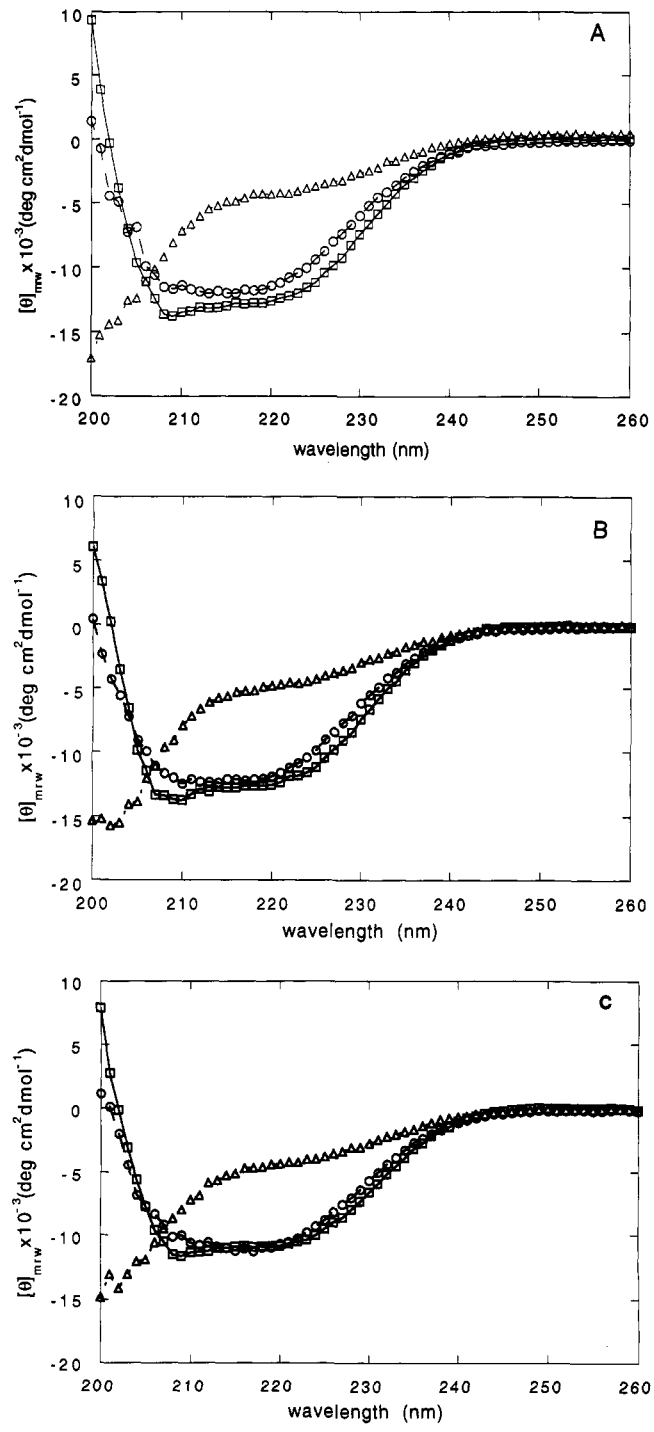

FIGURE 3: Far-UV CD spectra of different conformational states of β-lactamase and its derivatives as a function of pH. Panel A, wild-type protein: native (pH 6.5), open squares; acid-unfolded (U_A) (pH 1.0), triangles; A state (pH 0.5), open circles. Panel B, E166C mutant protein: native (pH 6.8), open squares; acid-unfolded (U_A) (pH 2.0), triangles; A state (pH 0.5), open circles. Panel C, spin-labeled E166C mutant: native (pH 6.6), open squares; acid-unfolded (U_A) (pH 2.0), triangles; A state (pH 0.5), open circles. Protein concentration was 15 μM.

unfolding transition and the acid-unfolding transition occurring at slightly higher pH. As shown in Figure 3C, the far-UV CD spectra of the spin-labeled derivative of E166C in the acid-unfolded state and in the A stage at pH 0.5 are similar to those of the wild-type protein and the unmodified E166C β-lactamase.

The solid lines in Figure 4 are curve-fits to eq 1 (where X^- represents the anion concentration, n and m are the number of ions bound, and K_1 and K_2 represent the binding constants) which is based on the assumption that the transition between

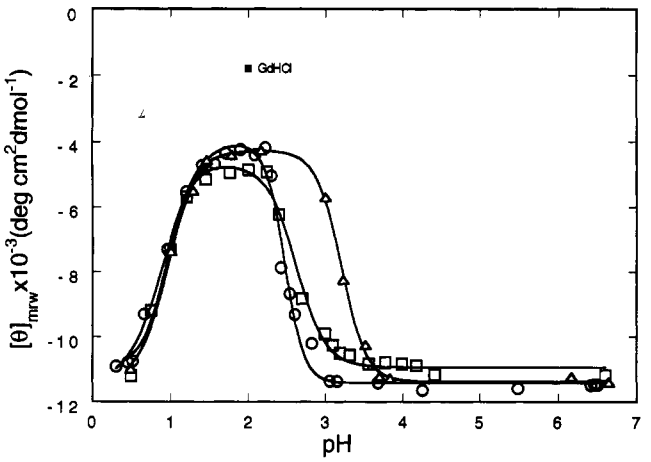

FIGURE 4: HCl titration of salt-free β-lactamase monitored by ellipticity at 222 nm. Wild-type protein is shown by squares, E166C by open circles, and the spin-labeled derivative of E166C by triangles. The solid lines are curve-fits calculated according to eq 1 (see text). The solid square represents the ellipticity for fully unfolded β-lactamase (pH 2, 6 M Gdn·HCl).

N and U_A and is dependent on a limited number of key protonations of carboxyl groups and the transition between

$$\theta_{obs} = \frac{\theta_{U_A}}{1 + \frac{[X^-]^n}{K_1} + \frac{K_2}{[H^+]^m}} + \theta_N \qquad (1)$$

U_A and A is dependent on the binding of a limited number of critical anions. The number of anions bound was found to be the same for all three proteins, 2.3 ± 0.3, and the number of protons bound was found to be 2.8 ± 0.5, also the same for all three species within experimental error.

We have previously shown that the formation of compact intermediate states at low pH can be accounted for as follows. As all the carboxyl groups of the protein become titrated, the net positive charge of the molecule leads to intramolecular charge repulsion, which leads to the observed acid unfolding transition in the vicinity of pH 3–4, corresponding to the pK of carboxylate. The subsequent addition of anions, either from increased acid concentration (as the pH is further lowered) or due to the addition of salt at pH 2, leads to anion binding and screening of the repulsive positive charges, allowing the intrinsic hydrophobic driving force for folding to collapse the molecule into the compact intermediate state (Goto et al., 1990b).

We examined the behavior of the *B. licheniformis* enzyme at pH 2, where it is in the acid-unfolded state, as a function of added KCl and sodium trichloroacetate. Both salts induced formation of the A state; trichloroacetate was far more effective than chloride, 10 mM being sufficient to cause the return of the native ellipticity at 222 nm (Figure 5A), whereas 400 mM chloride was required to regain nativelike secondary structure (Figure 5B). Sulfuric acid (0.3 M) also induced the A state, with a nativelike ellipticity at 222 nm (data not shown). Thus two different types of conditions were observed to yield the A state with maximum secondary structure: titration to pH 0.5 with HCl alone, or HCl titration to pH 2 plus salt. Confirmation that the protein under these sets of conditions was indeed in a compact intermediate state was obtained by examination of the near- and far-UV CD spectra (Figures 3 and 6) and measurements of the Stokes radius by gel filtration experiments (Table I). Whereas native *B. licheniformis* β-lactamase has a strong negative ellipticity in the 270–280-nm region due to the Tyr and Trp contributions and reflecting the native tertiary structure, both the A state and the acid-

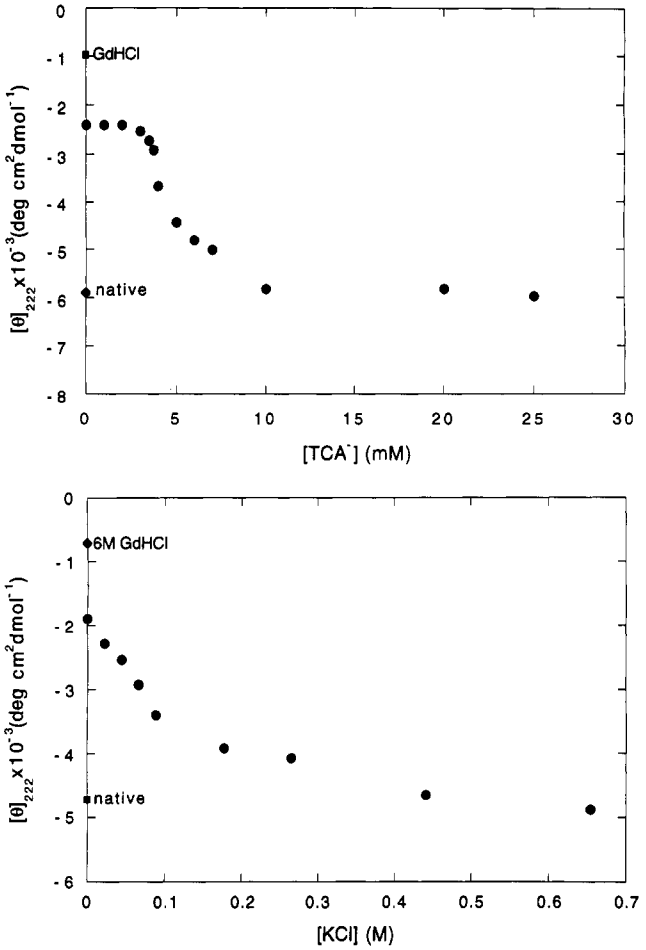

FIGURE 5: Anion-induced transitions of acid-unfolded β-lactamase to the A state. Anions were (panel A) trichloroacetate and (panel B) chloride. Conditions were pH 2.0. The transition was followed by ellipticity at 222 nm.

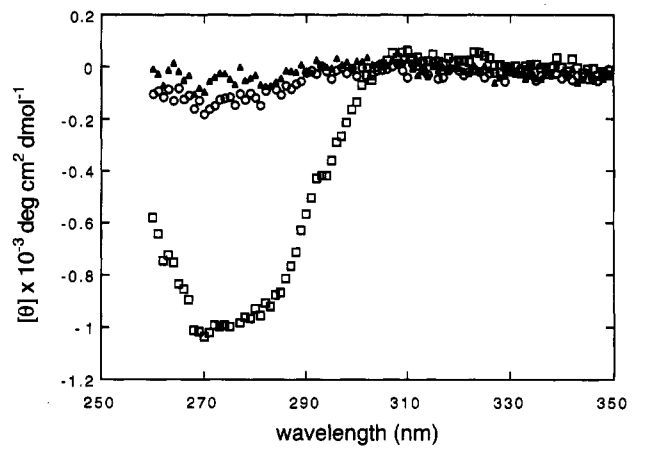

FIGURE 6: Near-UV CD spectra of the native, acid-denatured, and A states of the spin-labeled derivative of E166C. The native state is shown by squares, the acid-unfolded state, U_A, by triangles (pH 2.0), and the A state in 8 mM trichloroacetate, pH 2.0, by open circles.

unfolded state show no near-UV CD signal, consistent with the apparent loss of tertiary structure (Figure 6), while at the same time showing nativelike secondary structure as judged by the far-UV CD signal. The Stokes radius for the A state is expanded about 10% relative to that of the native state, compared to an expansion of about 100% for the unfolded protein, indicating that the A state is compact. No significant differences in the near-UV CD spectra for the different conformational states were noted between the spin-labeled derivative of E166C and the wild type.

Table I: Compactness of A State β-Lactamase, Relative to Native and Unfolded States

conformational state	R_s[a]	relative compactness[b]
native[c]	23.6	1.0
unfolded	51.0	2.2
A state[c]	25.6	1.1

[a] Stokes radius, in angstroms, as determined from size-exclusion chromatography. The estimated error is ±1 Å. [b] Hydrodynamic radius relative to that of native = 1.0. [c] The native states of the wild type, E166C, and spin-labeled E166C had identical retention volumes. Similarly, within experimental error, the A states of all three proteins had identical retention volumes.

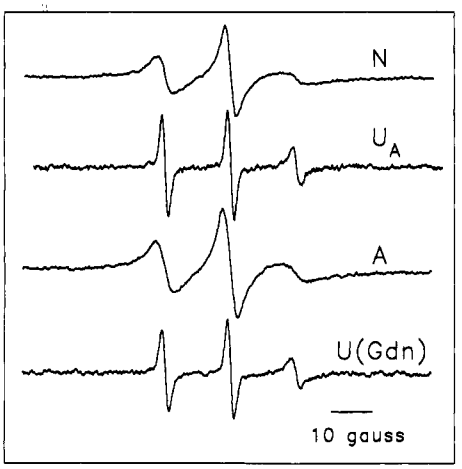

FIGURE 7: Esr spectra of spin-labeled E166C β-lactamase in different conformations. Spectrum N is the native state, pH 7.0. Spectrum U_A is the acid-unfolded state, pH 2.0. Spectrum A is the A state, pH 2.0, 8 mM trichloroacetate. Spectrum U is the spectrum of the fully unfolded protein in 6 M Gdn·HCl, pH 2.0. Protein concentration was 15 μM.

As mentioned previously, a characteristic of compact intermediate states such as the A state is the presence of exposed hydrophobic surface. This frequently leads to a marked propensity for aggregation. The β-lactamase A state, both wild type, mutant, and spin-labeled derivative, is no exception, as discussed below.

Esr Spectra of Spin-Labeled β-Lactamases. Esr spectra were collected for the spin-labeled Cys-166 derivative under various conditions. The spectrum of the native state was obtained at pH 7.0, 25 °C, that of the fully unfolded protein in 4 M Gdn·HCl, and for the acid-unfolded state in HCl at pH 2. Spectra of the A state were collected at pH 2, 0.3 M KCl; at pH 2, 8 mM and 20 mM TCA; and at pH 0.5 and 0.7 in HCl. In addition the effect of temperature was examined for the A state at pH 0.5, and at pH 2 with 8 mM TCA in order to examine the effect of temperature on aggregation.

The esr spectrum of the A state formed at pH 2, with 8 mM TCA, 25 °C, is compared to those of the native and unfolded protein in Figure 7. Analysis of the line widths of the spectrum of the native protein by spectral simulation indicated the rotational correlation time, τ_R, was = $(1.7 \pm 0.2) \times 10^{-9}$ s. The line widths for the acid-unfolded state are noticeably narrower than those for the native state, reflecting the greater mobility of the probe in the unfolded state, and yield a rotational correlation time of $(6.0 \pm 0.6) \times 10^{-10}$ s. The spectrum of the A state protein is essentially superimposable on that of the native protein and gives an identical τ_R [$(1.7 \pm 0.2) \times 10^{-9}$ s]. For comparative purposes the esr spectrum of the guanidine-unfolded state is also shown in Figure 7 and can be seen to be comparable to that of the acic-unfolded state. In this case the rotational correlation time was $(7.0 \pm 0.7) \times 10^{-10}$ s. These data indicate that the environment of the spin label in the A state is very similar to that in the native state and that it is much less mobile than in the unfolded state. We therefore conclude that there must be substantial structure remaining in the vicinity of residue 166 in the A state. This is rather surprising, given the apparent lack of tertiary structure as observed by other methods, and the fact that the A stage may be considered to be a compact *denatured* state (Dill & Shortle, 1991; Alonso et al., 1991). However, Ptitsyn and Semisotnov (1991) have reported that the molten globule state of spin-labeled α-lactalbumin also has an esr spectrum similar to that of the native state.

The A state has often been considered to be a molten globule, of which several different definitions, both explicit and implicit, have been used. The term was first used to describe a compact intermediate state of cytochrome *c* (Ohgushi & Wada, 1983) and was defined in some detail by Ptitsyn (1987). A theoretical description of the molten globule states has been given by Finkelstein and Shakhnovich (1989). The generally accepted properties of a molten globule include that it is a compact state containing substantial amounts of secondary structure and little or no nativelike tertiary structure. The compactness is assumed to involve very little expansion compared to the native state, perhaps a 10% increase in Stokes radius. The amount of secondary structure, as determined by far-UV circular dichroism, is comparable to that in the native state, and the fluctuating nature of the side chains will be apparent through the loss of the near-UV CD signal and fluorescence anisotropy and depolarization. The 1D ^{1}H NMR spectrum will closely resemble that of the unfolded state and there will be limited 2D NMR signals from nonbonded interactions (Baum et al., 1989). Other frequently found properties are a strong propensity to aggregate, especially at higher concentrations, and the ability to bind the fluorescent hydrophobic dye ANS; both these characteristics reflect increased exposure of hydrophobic surfaces in the molten globule state. It is also believed that there is no significant enthalpic or heat capacity difference between the molten globule and the unfolded state, as determined by calorimetry. The measured properties of the β-lactamase A state are therefore consistent with those of a molten globule on the basis of the above description.

The spin label in the E166C mutant is located in the active site, which also corresponds to the interface between the two major structural domains of the protein (Moews et al., 1990). This region is thus one which would be anticipated to be particularly likely to lose tertiary structure interactions in forming the molten globule conformation. The fact that the spin label senses an environment comparable to that in the native state implies that this region of the structure is still sufficiently compact to prevent significant increased mobility of the side chains. The hydrodynamic data indicate that the protein is slightly expanded in the A state. The tryptophan fluorescence emission maximum in the A state is similar to that in the native state (Goto & Fink, 1989) although significantly quenched. This suggests that the Trp residues are in a nonpolar, buried environment in the A state; the quenching is likely to be due to increased mobility of the side chains leading to dynamic quenching. There are few reasonable models which are consistent with the limited expansion, the nativelike far-UV CD spectrum, the loss of the near-UV CD signal, and the limited mobility of the spin label. One model involves small movements of the secondary structural components so as to pull the side chains apart sufficiently to result in loss of their interdigitation. That would account for the small expansion, the loss of the near-UV CD signal, the

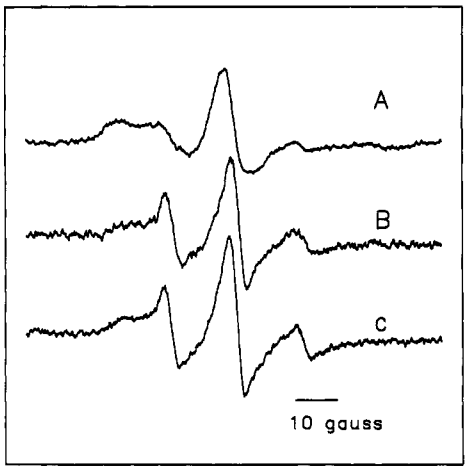

FIGURE 8: Esr spectra of the A state of spin-labeled E166C β-lactamase under conditions leading to aggregation. Spectrum A is at pH 2.0, 20 mM trichloroacetate; spectrum B at pH 2.0, 300 mM chloride; and spectrum C at pH 0.5. Protein concentration was 15 μM.

presence of the tryptophans in a nonpolar environment, and the retention of nativelike secondary structure as detected by far-UV CD. The esr results reported here could be interpreted in the light of this model as follows: since the spin label is larger than the wild-type side chain it will be in a more crowded environment than most side chains. The molecular expansion on forming the A state does not lead to sufficient increase in space in the local environment of the spin label to allow increased mobility. An alternate model is one in which the central core of the molecule remains nativelike, whereas peripheral regions undergo partial unfolding. This model does not adequately account for the loss of the near-UV CD signal, however. An alternative model would be one in which subdomains move apart slightly, leading to loss of the tertiary interactions between them, while maintaining secondary structure, some tertiary interactions (within subdomains), and leading to some expansion of the molecule. The spin label results would then be interpreted again in terms of the spatial crowding by the label not being relieved in the A state.

The sensitivity of esr line width to rotational correlation means that it is particularly sensitive to aggregation. This became manifest when the initial attempts to examine the A state esr spectrum were made, for example at pH 2 in the presence of 300 mM Cl^- (Figure 8). Procedures which were investigated to eliminate the aggregation included minimizing the protein concentration, varying the temperature, and changing the conditions for formation of the A state. The minimum satisfactory protein concentration for the esr signal was found to be 15 μM. Attempts to slow the aggregation process down by decreasing the temperature were not very satisfactory due to increased esr line widths and limited effects on the rate of aggregation. Optimal conditions at 25 °C were found to be pH 2.0 and 8 mM TCA. Higher concentrations of TCA tended to increase the propensity for aggregation. The spectrum of the A state obtained under these optimal conditions is shown in Figure 7. A spectrum obtained at higher TCA concentration, 20 mM, is shown in Figure 8, and the contribution of aggregation can readily be seen in both the increased line widths and the broad line appearing at low field. Esr spectra of the A state in the presence of chloride, either at pH 2 and 300 mM KCl (or higher) or at pH < 0.7, showed evidence of aggregation, even though that was no evidence of it in the CD spectra. Given that the rotational correlation time of the protein is considerably longer than that of the spin labeled in the nonaggregated case, the observed line broadening indicates that the aggregation involves protein–protein interactions which decrease the mobility of the spin label.

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