Characterization of the Unfolding Pathway of Hen Egg White Lysozyme†

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ABSTRACT: After the recent discovery of a ribonuclease A unfolding intermediate [Kiefhaber, T., *et al.* (1995) *Nature 375,* 513−515], we investigated the unfolding pathway of hen egg white lysozyme. At pH* 4.00 with D_2O at 10 °C and 6 M guanidinium chloride, unfolding shows a single, slow kinetic phase, with a relaxation time of 3300 s when monitored by circular dichroism (CD). Exchange of the tryptophan indole nitrogen protons shows that buried Trp residues 123, 111, and 108 lose tight packing and become solvent-exposed simultaneously, with a mean relaxation time of 3300 s, similar to the CD-monitored unfolding rate. Unfolding monitored by Trp fluorescence shows, moreover, that 90% of the amplitude change occurs in a slow phase, with a relaxation time of 2400 s. Faster-unfolding phases with minor amplitudes are detected by Trp indole hydrogen exchange and by fluorescence. It is likely that these changes are caused by Trp 62 and Trp 63, active site residues which are not buried in the hydrophobic core. Lysozyme unfolding was further monitored by the histidine 15 Cϵ1 proton, which gives resolved lines for the native and unfolded species in one-dimensional ^{1}H-NMR spectra. The majority of the unfolding reaction, 70%, occurs in a slow phase with a relaxation time of 3600 s, but there is also a rapid unfolding phase; 30% of the His 15 Cϵ1 proton resonance intensity is found at the unfolded chemical shift within tens of seconds after the start of unfolding. The amplitude of the rapid unfolding phase increases proportionally with the concentration of GdmCl denaturant present. These results show that a partially buried residue of lysozyme, histidine 15, takes part in forming an unfolding intermediate similar to the one observed earlier for valine 63 in ribonuclease A. The tryptophan side chains buried in the hydrophobic core of lysoyzme, in contrast, do not participate in forming the unfolding intermediate, as judged by proton chemical shifts. The buried tryptophan residues of dihydrofolate reductase, monitored by ^{19}F-NMR, do participate in forming an unfolding intermediate [Hoeltzli, S. D., & Frieden, C. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92,* 9318−9322]; the difference between that study and ours may reside in the greater sensitivity of ^{19}F to the detection of motional differences.

Kinetic studies of the unfolding reactions of globular proteins monitored by CD[1] or fluorescence show that proteins typically unfold in a single kinetic step, N → U, with no detectable intermediates (Schmid, 1992; Mücke & Schmid, 1994). Thus, it was surprising when Kiefhaber *et al.* (1995) discovered an intermediate in the GdmCl-induced unfolding of RNase A. Using 1D ^{1}H-NMR, which is sensitive to subtle environmental changes, they observed a fast decrease in the intensity of the Val 63 CγH resolved spectral line. They interpreted the results to mean that the unfolding RNase A forms an intermediate with intact secondary structure in which side chains lose tight packing but remain solvent-shielded. More recently, Hoeltzli and Frieden (1995) used stopped-flow ^{19}F-NMR to show that dihydrofolate reductase (DHFR) forms an unfolding intermediate with similar properties. Both the ribonuclease A and DHFR unfolding intermediates are similar to the dry molten globule form proposed on theoretical grounds by Shakhnovich and Finkelstein (1989).

These results are startling. To test the generality of this behavior, we decided to study the unfolding of hen egg white lysozyme. Hen egg white lysozyme is composed of two domains: an α-domain that contains several helices and a β-domain comprised largely of β-strands (Figure 1). Lysozyme has many desirable characteristics for this work; its NMR assignments are known (Redfield & Dobson, 1988), and its refolding behavior has been studied in depth (Kiefhaber, 1995; Itzhaki *et al.,* 1994; Dobson *et al.,* 1994). Lysozyme unfolds very slowly, making the study of unfolding by 1D ^{1}H-NMR practical. Five of the protein's six tryptophan indole nitrogen protons give unique lines in 1D ^{1}H-NMR spectra. Upon denaturation, these lines disappear and the indole protons resonate in a common line at 10.06 ppm. Moreover, when the Trp side chain is exposed to D_2O, the indole proton is exchanged for a deuteron and the NMR line disappears. Thus, the tryptophan residues can be used to probe two steps of unfolding: the disordering of side chains and their exposure to solvent. In addition, lysozyme contains a single histidine residue (Figure 1). At low pH, the resonance lines of the Cϵ1 proton of this histidine in the folded and unfolded states are well-resolved. By measuring the intensity of each line during unfolding, we can quantitate the kinetics of the unfolding reaction. As a complement to the NMR experiments, fluorescence and UV absorbance methods were used to monitor the bulk unfolding behavior

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[1] Abbreviations: CD, circular dichroism; 1D, one-dimensional; C_m, the midpoint of the protein unfolding transition; DHFR, dihydrofolate reductase; $\Delta G(H_2O)$, free energy of stability extrapolated to zero denaturant concentration; GdmCl, guanidinium chloride; *m*, *m* value (the steepness of the unfolding transition); NaAc, sodium acetate; lysozyme, hen egg white lysoyzme; pH*, the apparent pH meter reading without the deuterium isotope effect correction; RNase A, ribonuclease A from bovine pancreas; TSP, trimethyl silyl propionate.

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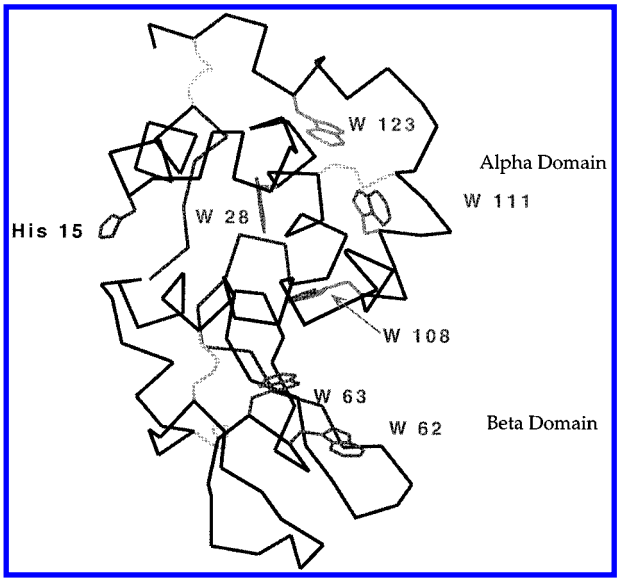

FIGURE 1: Hen egg white lysozyme. A Cα backbone trace and the histidine, tryptophan, and cystine residues are shown (PDB file 8LYZ; Beddell *et al.*, 1975).

of aromatic residues, and CD was used to monitor the unfolding of secondary structure.

MATERIALS AND METHODS

Reagents. The guanidinium chloride and urea were the ultrapure grade from Gibco BRL. D_2O (99.9% D) was purchased from Cambridge Isotope Laboratories. Sodium chloride, sodium acetate, and hydrochloric acid were obtained from J. T. Baker, while deuterium chloride (99.9% D) was from Isotech Corp. The protein used was hen egg white lysozyme (lysozyme) (EC 3.2.1.17, Sigma L-6876 lot 53H7145).

Protein Preparation. Typically, 200 or 300 mg of lysozyme was dissolved in double-glass-distilled water acidified with 1 mL of 5 M HCl. The protein was dialyzed against 2 L of double-glass-distilled water acidified with 1 mL of 5 M HCl. This buffer was changed three times. Afterward, aliquots were taken, freeze-dried, and stored at −20 °C. To follow unfolding by the His 15 Cϵ1 proton, amide protons were first exchanged for deuterons by dissolving the protein in D_2O and heating to 80 °C for 15 min. The protein was left to cool at room temperature for 1 h and then was freeze-dried. This process of dissolving in D_2O, heating, and freeze-drying was repeated.

Experimental Conditions. The standard conditions for characterizing the native state were 100 mM NaCl and 50 mM NaAc at in pH* 4.00 in D_2O at 10.0 °C. Final unfolding conditions were 100 mM NaCl, 50 mM NaAc, and 6.00 M GdmCl at pH* 4.00 in D_2O at 10.0 °C. Concentrations of GdmCl were measured by weight and by refractive index (Pace *et al.*, 1989). Unfolding was initiated by dilution of lysozyme dissolved in native buffer into denaturant solution. For some NMR experiments, unfolding was initiated by direct addition of denaturant to lyophilized protein. Similar unfolding results were obtained by both of these methods. Other experiments, not detailed here, were performed at different pH* values, temperatures, and salt and GdmCl concentrations. These results were used to make small pH* interpolations to the standard pH* value of 4.00.

Circular Dichroism. An Aviv model 60DS spectropolarimeter equipped with a Peltier temperature control unit was used for all CD measurements. Equilibrium spectra were the average of five scans recorded in 0.2 nm steps with a 1.0 nm band width at 10.0 °C, while kinetic unfolding spectra were either one or the average of two scans recorded in 0.5 nm steps. Kinetic changes monitored at a single wavelength were recorded using a 5 s averaging time.

UV Absorbance. UV absorbance was recorded using a Cary 60 dual-beam UV/Vis spectrometer. Equilibrium difference spectra were recorded using a pair of matched tandem cuvettes; the sample cell contained protein and denaturant in the first compartment and buffer in the second. In the reference cuvette, buffer and protein were placed in the first compartment and denaturant was placed in the second. Difference spectra were scanned at 0.1 nm/s. Spectra were recorded at room temperature or 13 °C. Unfolding kinetics were followed at 301 nm and 10 °C.

Fluorescence. An Aminco·Bowman series 2 luminescence spectrometer was used for fluorescence measurements. Native lysozyme showed a broad emission at 333 nm. The fluorescence emission of unfolded protein was enhanced and red-shifted to a maximal emission at 347 nm. Excitation at 295 nm assured that only Trp residues are excited, facilitating structural interpretation. A narrow excitation slit width of 2 nm was used to minimize photobleaching. Unfolding kinetics were followed as emission at 350 nm. Stopped-flow kinetics were followed using an Applied Photophysics apparatus, using an excitation wavelength of 295 nm and monitoring total emission above 305 nm.

Nuclear Magnetic Resonance Spectroscopy. All experiments were performed using a General Electric GN-Omega spectrometer, operating at 500 MHz. The magnet was tuned and shimmed with a sample identical in volume and composition to the sample used in the unfolding sample. Unfolding was initiated by adding 500 μL of denaturant solution directly to the dry lyophilized protein powder or to protein dissolved in a small amount (50 μL) of native buffer. After gentle mixing, the solution was loaded into the NMR tube and placed in the magnet. The sample was shimmed briefly before starting acquisition of sequential 1D NMR spectra of 64 scans each (4096 real points, spectral width of 7000 Hz). For each unfolding spectrum, the midpoint of the data acquisition was used as the time value. The dead time of this manual-mixing procedure was 2−3 min. To decrease this dead time, we also initiated unfolding by using a syringe to inject denaturant directly into a solution of lysozyme and native buffer already placed inside the NMR magnet. After injection, sequential acquisition of spectra was started immediately without shimming or tuning. The dead time of this experiment was about 10 s, and as only 16 or 32 scans with a recycling delay of 1 s were recorded per spectra, the first spectrum was completely acquired only 25 s (16 scans) after the start of unfolding. All chemical shifts are reported relative to TSP as an internal standard.

For the lysozyme spectrum in H_2O (Figure 4a), 3072 acquisitions were recorded and the water peak was suppressed by presaturation. We also followed the unfolding of lysozyme in protonated 6 M GdmCl solution, to measure the increase of the unfolded Trp indole proton peak during unfolding. For this experiment, 128 acquisitions using presaturation of the water line were recorded per spectrum. A quench experiment was also performed to test if some of the lysozyme Trp side chains become exposed early during unfolding. In this experiment, deuterated lysozyme was briefly unfolded in protonated 6.00 M GdmCl solution at

10.0 °C. Unfolding and exchange were quenched by an 11-fold dilution with D_2O buffer.

Software. NMR data were processed using Felix, version 2.30, from Biosym Technologies (San Diego). The FID was multiplied by a shifted sine bell curve prior to Fourier transformation. The baselines were corrected by fitting to a zero-order polynomial function. NMR peak heights and areas were measured by simulation using the Felix peak optimization subroutine. Both peak heights and peak areas were used to determine kinetic fits and yielded similar kinetic parameters. Kaleidagraph, version 3.0.1 (Abelbeck Software, Reading, PA), was used to process and fit kinetic data and to prepare graphs.

RESULTS

Equilibrium Unfolding. The equilibrium stability of lysozyme unfolded by GdmCl was measured under the conditions used for the kinetic unfolding experiments. The unfolding transition followed by circular dichroism was analyzed using the two-state model (Santoro & Bolen, 1988; Pace *et al.*, 1989) (Figure 2A), yielding $\Delta G(H_2O) = 8.2$ kcal/mol, $m = 2000$ cal M^{-1} mol^{-1}, and $C_m = 4.19$ M. These data confirm that lysozyme is completely unfolded in 6 M GdmCl. Similar unfolding midpoints were obtained when unfolding was followed by fluorescence spectroscopy or by 1D ^{1}H-NMR measurement of histidine and native alkyl resonances (data not shown), suggesting that the unfolding transition of lysozyme is close to two-state under the experimental conditions used for the kinetic unfolding experiments.

Circular Dichroism-Monitored Unfolding. When lysozyme unfolding is followed kinetically at 222 nm, a single unfolding reaction is observed (Figure 2B). The amplitude of this reaction is 7900 deg cm^2 $dmol^{-1}$ $residue^{-1}$, which is close to the change of 8100 deg cm^2 $dmol^{-1}$ $residue^{-1}$ (mean of three experiments) observed from equilibrium unfolding experiments (Figure 2A).

The recording wavelength (222 nm) is close to the isodichroic point given by native lysozyme and the refolding intermediate (formed after 4 ms of refolding) that was studied by Chaffotte *et al.* (1992). Because refolding and unfolding intermediates might have similar spectral properties, unfolding was also followed at 227 nm. This wavelength was chosen because it maximizes the signal difference between native protein and the early-refolding intermediate. The results at 227 nm for unfolding are very similar to those recorded at 222 nm; only one kinetic phase, with a k_{obs} of $(3.0 \pm 0.4) \times 10^{-4}$ s^{-1} (repeated twice), is observed, and this phase accounts for all the amplitude difference between the native and denatured states at this wavelength, as estimated from equilibrium unfolding experiments (Figure 2A).

Chaffotte *et al.* (1992) also recorded the CD spectrum of the early intermediate during the refolding of lysozyme. The spectrum of this intermediate is different from that of native lysozyme but is very similar to a calculated spectrum that includes only lysozyme's secondary structure and neglects the contribution of side chains. The authors concluded that this refolding intermediate has native-like secondary structure but lacks packed side chains. With this in mind, we recorded CD spectra during the unfolding reaction to test the cooperativity of unfolding (data not shown). In addition, unfolding spectra expected for a cooperative two-state transition

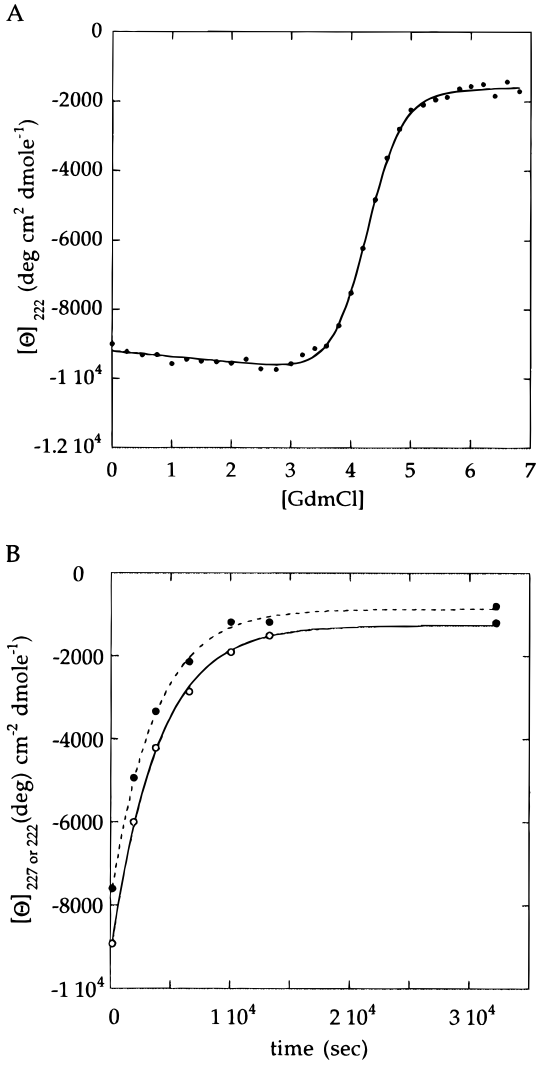

FIGURE 2: Unfolding of lysozyme monitored by CD. (A) Equilibrium denaturation of lysozyme by GdmCl. Lysozyme was unfolded at pH* 4.0 in 6.00 M GdmCl, 100 mM NaCl, and 50 mM NaAc at 10.0 °C in D_2O. Unfolding was monitored by CD at 222 nm. Data points are indicated by filled circles, and the solid curve-fitted by least-squares (Santoro & Bolen, 1988), which gave a $\Delta G(H_2O)$ of 8.20 kcal/mol and an m value of 1900 cal mol^{-1} M^{-1}. (B) Unfolding kinetics of lysoyzme monitored by far-UV CD. Unfolding was followed by CD at 222 nm (open circles) and 227 nm (filled circles). The curves (222 nm, solid; 227 nm, broken) indicate fits to a single-exponential equation. The unfolding conditions are as follows: pH* 4.0, 6.00 M GdmCl, 100 mM NaCl, and 50 mM NaAc, and in D_2O at 10.0 °C.

were calculated from spectra of native and fully unfolded lysozyme, using the fractions of native and unfolded protein present at a given time during unfolding. These model spectra are in close agreement with the experimental spectra (data not shown), indicating that the loss of native secondary structure and tertiary packing of aromatic and cystine residues during unfolding is cooperative.

Unfolding Monitored by Ultraviolet Absorbance. To determine the absorbance change that accompanies complete unfolding, a difference spectrum was recorded for lysozyme unfolded in 6 M GdmCl versus lysozyme in native buffer. A broad difference peak, with a maximum at 301 nm, was observed (data not shown). The absorbance difference at 301 nm corresponds to an extinction difference of 1250 cm^{-1} M^{-1}, which we take as the total amplitude change expected for unfolding. Monitored by ultraviolet absorbance at 301 nm, lysozyme was found to unfold with an observed rate

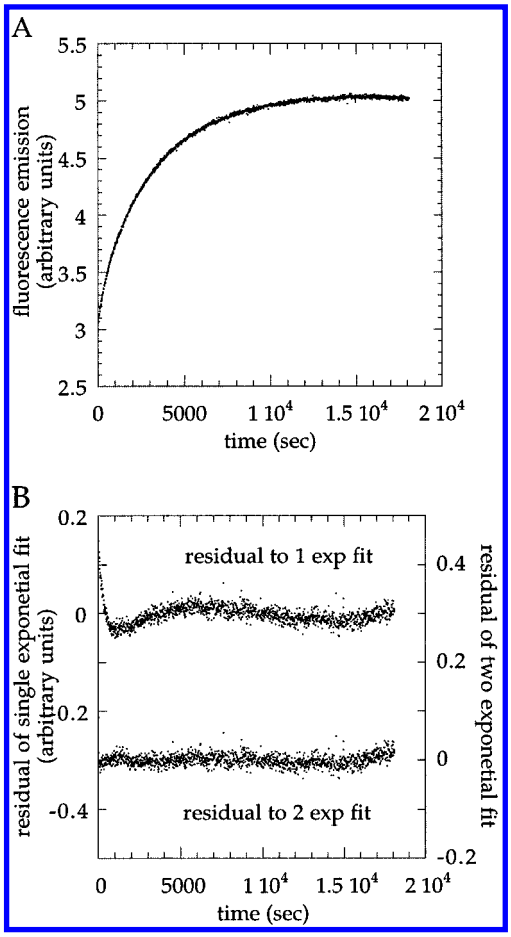

FIGURE 3: Unfolding kinetics of lysozyme followed by ultraviolet fluorescence emission. (A) Lysozyme unfolding at pH* 4.0 in 6.00 M GdmCl, 100 mM NaCl, and 50 mM NaAc in D_2O at 10.0 °C. The excitation wavelength was 295 nm, and the emission wavelength was 350 nm. The experimental data are fitted by a two-exponential equation. (B) The residual plots to one- and two-exponential fits of this data.

constant of $(1.7 \pm 0.8) \times 10^{-4}$ s^{-1} (mean of two experiments) and an extinction coeffecient change of 900 ± 80 cm^{-1} M^{-1} (mean of two experiments). The error of these kinetic parameters is larger than the values given because of a slow signal drift. This signal drift is probably the result of photobleaching of the unfolded protein.

Fluorescence-Monitored Unfolding. Lysozyme unfolding was followed by UV fluorescence spectroscopy, using an excitation wavelength of 295 nm, which selectively excites the Trp residues (Figure 3A). The unfolding data were found to be fairly well described by a single-exponential decay, but the fit to a two-exponential decay was substantially better (Figure 3B). The two-exponential fit gave unfolding rates of $(3.4 \pm 0.1) \times 10^{-3}$ and $(4.1 \pm 0.1) \times 10^{-4}$ s^{-1}, with fractional amplitudes of 0.10 ± 0.03 and 0.90 ± 0.03, respectively (mean values of two experiments). The amplitude of the kinetic unfolding experiment was only about 70% of that of the equilibrium unfolding experiment performed on the same day (repeated twice). This suggested the possibility that a third, very rapid unfolding phase might be present to account for the missing kinetic unfolding amplitude. Such a fast phase was not, however, detected by stopped-flow fluorescence (data not shown) (see Note Added in Proof). Tanford *et al.* (1973) observed two kinetic phases in the unfolding of lysozyme at pH 2.6 monitored by ultraviolet absorbance at 301 nm.

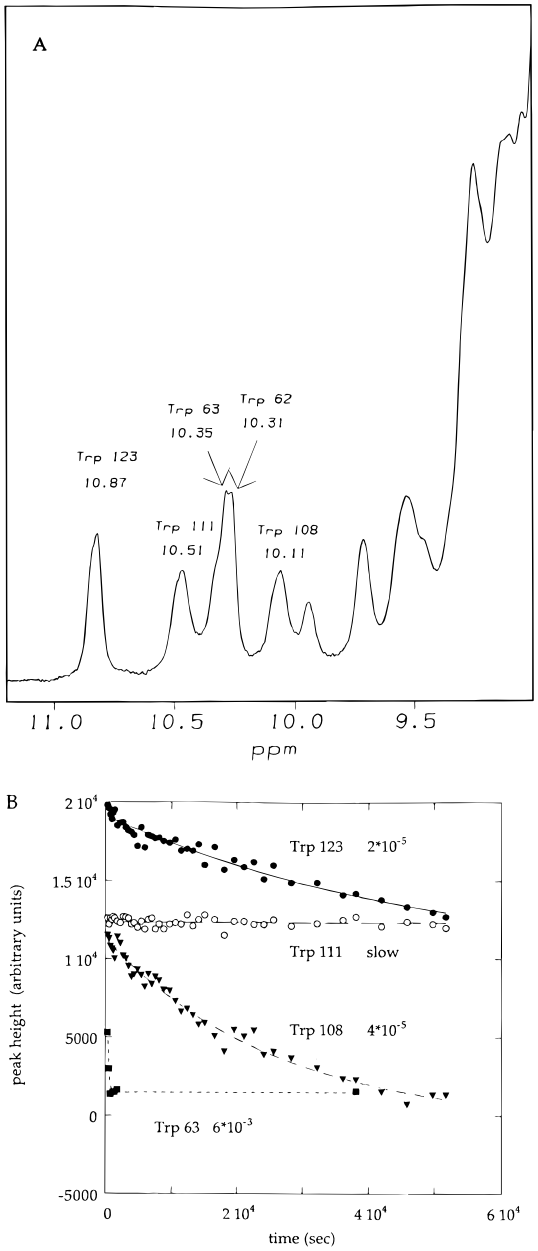

FIGURE 4: Native state hydrogen exchange of lysozyme tryptophan indole nitrogen protons. (A) 1D ^{1}H-NMR spectrum of lysozyme recorded in H_2O at pH 4.0 with 100 mM NaCl, 50 mM NaAc, and no GdmCl at 10 °C showing the presence of the Trp indole nitrogen proton peaks. (B) The exchange rates of the Trp indole nitrogen protons from folded lysozyme in D_2O at pH* 4.0 with 100 mM NaCl, 50 mM NaAc, and no GdmCl at 10 °C. The identity and exchange rate in units of s^{-1} are given.

Unfolding Monitored by Trp Indole Proton Exchange. To follow the unfolding of the individual Trp residues, we turned to 1D ^{1}H-NMR spectroscopy. Five of lysozyme's six Trp indole protons have unique lines in NMR spectra. The exception is Trp 28, whose resonance at 9.35 ppm overlaps with amide proton resonances. A NMR spectrum of lysozyme recorded in H_2O is shown in Figure 4A. The resonances of Trp 123 (10.87 ppm), Trp 111 (10.51 ppm), Trp 63 (10.35 ppm), Trp 62 (10.31 ppm), and Trp 108 (10.11 ppm) were assigned previously by Redfield and Dobson (1988). The line at 9.75 ppm is an amide proton resonance. NMR spectra of lysozyme in increasing concentrations of GdmCl were recorded to extend the Trp assignments to high GdmCl concentrations, the conditions of the unfolding experiments (data not shown). The native Trp lines generally

shift upfield with increasing GdmCl concentrations, becoming on average 0.1 ppm lower in 6 M GdmCl as compared to buffer. Unfolded in 6 M GdmCl (H_2O), the Trp protons were found to resonate in a single line with a shoulder at 10.04 ppm (data not shown). By recording a series of 1D ^{1}H-NMR spectra of protonated lysozyme at our standard experimental conditions in the absence of denaturant, we determined the exchange rates of the Trp indole protons in the native state (Figure 4B). Trp 111 showed very little exchange over a 14 h period, while Trp 123 and Trp 108 exchanged slowly with observed rates of 2×10^{-5} and 4×10^{-5} s^{-1}, respectively. Trp 63 exchanged rapidly with a k_{obs} of 6×10^{-3} s^{-1}, while Trp 62 exchanged so rapidly that its rate could not be measured. The very rapid exchange of Trp 62 is not unexpected; Wüthrich (1986) reported a rate of 1.3 s^{-1} for Trp indole nitrogen proton exchange at 25 °C and pH 4. To determine this value in our experimental conditions, the peptide Ac-Ala-Ala-Trp-Ala-Ala-NH_2 was prepared as a model for Trp in the unfolded protein and its indole proton exchange rate was measured to be approximately 0.02 s^{-1} at 10 °C and pH* 4 in 6 M GdmCl (data not shown). Thus, the exchange rate of an exposed Trp indole proton is very fast compared to the unfolding rate of lysozyme measured by CD. The behavior of the Trp indole proton resonances during exchange can, therefore, detect both the loss of tight packing (measured as chemical shift changes) and exposure of these residues to solvent (measured as the disappearance of the resonances through exchange). This technique should, therefore, provide clear evidence for or against the possible presence of a dry molten globule unfolding intermediate.

Unfolding of protonated lysozyme was monitored under our standard conditions, and NMR spectra recorded after initiation of unfolding are shown in Figure 5A. Lines with native chemical shifts of Trp 123, 111, and 108 are visible throughout the unfolding process. These three lines disappear slowly. In contrast, the small peak with a chemical shift of 10.20 ppm is present in the first several spectra but disappears more rapidly with a rate of $(1.7 \pm 0.4) \times 10^{-3}$ s^{-1} (mean of two experiments), which is similar to the rate of exchange exhibited by Trp 63 in folded lysozyme (Figure 4B). At 10.20 ppm, this peak lies close to the native resonance of Trp 63 but is also near the value expected for a Trp residue in the unfolded state, as measured by Bundi and Wüthrich (1979). Might a Trp residue in the dry molten globule state be the source of this resonance?

To test this possibility, we repeated the unfolding experiment with lysozyme that had been selectively deuterated at the Trp 63 indole nitrogen by incubating lysozyme in deuterated buffer for 30 min, taking advantage of the rapid exchange of the Trp 63 indole proton from native lysozyme (Figure 4B). The spectrum of unfolded lysozyme with preexchanged Trp 63 is shown in spectrum D of Figure 5A. No line is present at 10.20 ppm. Therefore, the line at 10.20 ppm in the original unfolding experiment is assigned to Trp 63. The buried Trp residues, 108, 111, and 123, unfold and exchange slowly (Figure 5B), at a mean rate of $(3.0 \pm 0.4) \times 10^{-4}$ s^{-1} (two experiments), similar to that observed by CD (Figure 2B). The Trp 108, 111, and 123 native resonances show no chemical shift changes, nor are their peak widths altered significantly during the unfolding process, indicating that these residues remain tightly packed in their native-like magnetic environments prior to the global unfolding reaction.

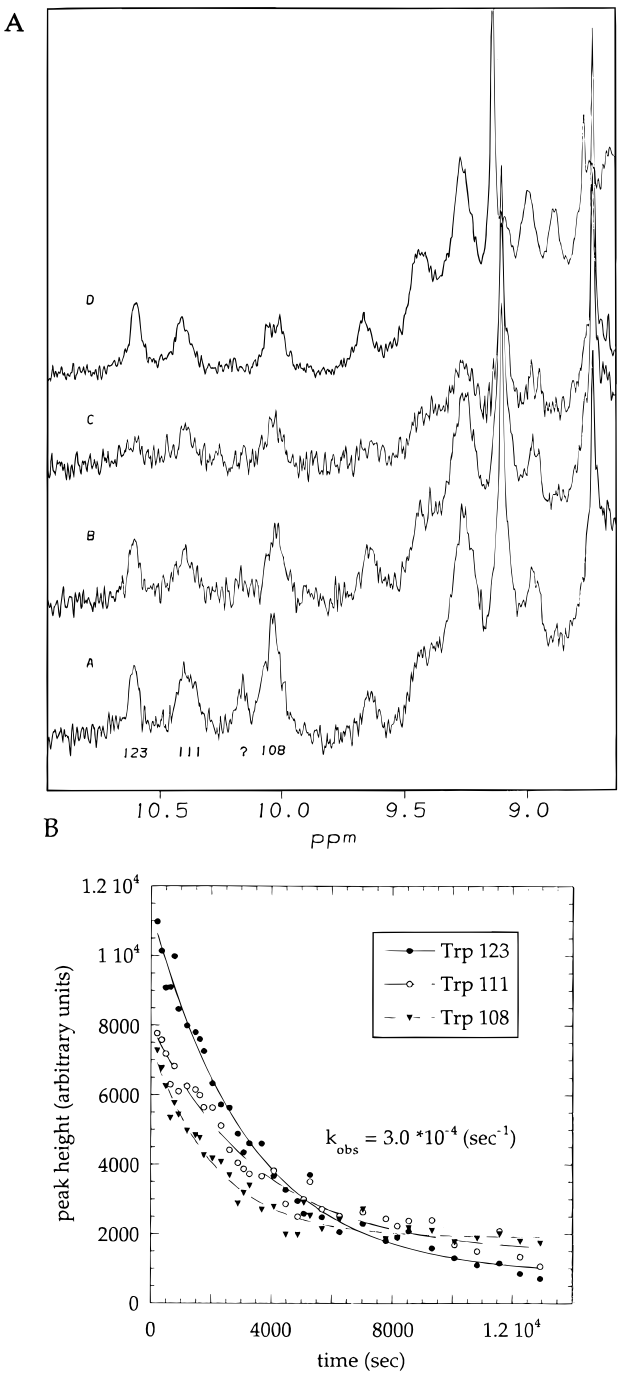

FIGURE 5: Kinetics of lysoyzme unfolding monitored by Trp indole nitrogen proton exchange. (A) Representative NMR spectra recorded at 4 min (spectrum A), 17 min (spectrum B), and 94 min (spectrum C) during unfolding at pH* 4.0 with 6.00 M GdmCl, 100 mM NaCl and 50 mM NaAc at 10.0 °C. An NMR spectrum of lysozyme selectively deuterated at Trp 63 recorded early during unfolding is shown in spectrum D. Note that there is now no peak at 10.2 ppm. The identities of the Trp residues are given below the spectrum A. (B) Kinetics of Trp indole nitrogen proton exchange, using lysozyme with the Trp 63 proton preexchanged for a deuteron. The average exchange rate of Trp 123, Trp 111, and Trp 108 is $(3 \pm 2) \times 10^{-4}$ s^{-1}.

To detect a possible early unfolding phase, we monitored the unfolding of lysozyme by 1D ^{1}H-NMR at pH 4.0 and 10.0 °C with 6.00 M GdmCl, 100 mM NaCl and 50 mM NaAc, and D_2O was replaced by a mixture of 90% H_2O/10% D_2O. This allowed a possible unfolding burst to be measured, as in H_2O the six unfolded indole protons are not lost to exchange but are altered in their chemical shift as

they lose their distinct magnetic environments. Throughout unfolding, the peak areas of native Trp resonances decrease and that of the composite unfolded Trp resonance at 10.04 ppm (which is superimposed on the native Trp 108 resonance at 10.06 ppm) increases (data not shown). The area of each Trp peak versus time was fitted to a single-exponential equation (data not shown). When this fit was extrapolated back to the start of unfolding, the peak areas were as follows: Trp 123, 1.1; Trp 111, 1.0; Trp 63 and 62, 0.7; and Trp 108 and unfolded Trp, 1.6 (normalized to the Trp 111 peak area). The sums of the areas for Trp 63 and 62 and for Trp 108 and unfolded Trp are given, rather than individual values, as these peaks overlap in the unfolding spectra. It is notable that the Trp 63 and 62 peak is smaller than a single Trp peak, rather than having a combined area of two. Also, a larger than expected area, 1.6 Trp, is found for the Trp 108 and unfolded Trp at the start of unfolding. These data suggest that some fraction of the Trp 63 or Trp 62 side chains or both unfold in a rapid burst phase.

We also employed a pulse/quench experiment to detect burst unfolding by exchange labeling the indole nitrogens that may become unfolded and solvent exposed early during unfolding with protons. Lysozyme, exhaustively deuterated at the indole nitrogens and predissolved in D_2O buffer, was unfolded in 6.00 M protonated GdmCl in H_2O buffer for 1 and 5 min. If the lysozyme Trp residues unfold during this time, their indole nitrogens should be labeled with protons, as hydrogen exchange is rapid ($k_{ex} \approx 10^{-2}$ s^{-1}) under these conditions. Unfolding and exchange were quenched by 11-fold dilution with D_2O buffer at pH* 5 on ice. This pH* value and the lower temperature were chosen to reduce the hydrogen exchange rate from that of the native state, which is already very slow for Trp 108, 111, and 123 (Figure 4B). The NMR spectra of these samples show no proton labeling in either spectrum, indicating that little or no solvent exposure of the buried lysozyme indole nitrogen protons occurs in the first 5 min of unfolding. Although formation of the dry molten globule species would not be detected by this experiment, this experiment does show that no buried lysozyme Trp residue becomes unfolded and solvent exposed in the first minutes of unfolding. A control experiment revealed that, after 24 h of unfolding in protonated 6 M GdmCl, the Trp indole nitrogens do become extensively proton labeled (data not shown).

Unfolding Monitored by 1D ^{1}H-NMR of Histidine 15. Lysozyme unfolding was also monitored by the appearance and increase of the unfolded His 15 Cϵ1 proton resonance at 8.78 ppm and the corresponding decrease of the native His 15 Cϵ1H line at 9.15 ppm. Representative spectra of lysozyme recorded at different times in the unfolding reaction are shown in Figure 6A. While the majority of the His 15 population unfolds slowly, with the same rate as the loss of helical structure detected by CD, a substantial amount of the protein appears unfolded in the first spectrum recorded after the initiation of unfolding. The fractional amplitude of the unfolded peak after 3 min of unfolding is 0.30. From the CD-monitored unfolding results, only 4% of the protein is expected to be unfolded at this time point. Following this burst of unfolding, the native peak slowly disappears and a peak indicative of the unfolded state gradually increases (Figure 6B). In our standard experimental conditions, unfolding of His 15 has a rate similar [$(2.8 \pm 1) \times 10^{-4}$ s^{-1}, mean of five experiments] to that of the unfolding structural changes detected by other techniques. We repeated

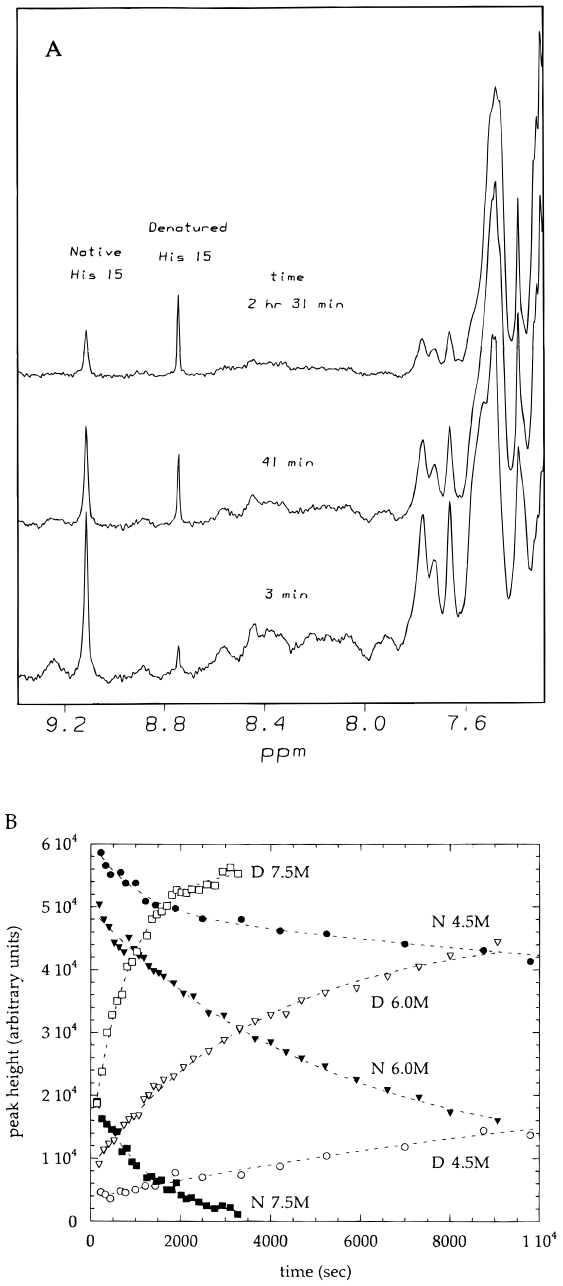

FIGURE 6: Unfolding kinetics of His 15 monitored by 1D ^{1}H-NMR. (A) Representative spectra at three different time points are shown. Both the native His 15 Cϵ1 proton peak, at 9.16 ppm, and the unfolded peak, at 8.75 ppm, are indicated. The times given are the midpoint of the acquisition of the spectrum. The unfolding conditions are as follows: pH* 4.3, 6.0 M GdmCl, 100 mM NaCl, and 50 mM NaAc, and in D_2O at 10 °C. (B) Peak heights of the native (N, filled symbols) and denatured (D, open symbols) resonances during unfolding. Three different denaturant concentrations (4.5, 6.0, and 7.5 M GdmCl) are shown. The curves are least-squares fits to an exponential function. Note that the denatured peak height is non-zero at time zero; this is the burst amplitude. Also note that the burst amplitude increases at higher GdmCl concentrations. The observed rates for this experiment are 1.9 and 2.6 $\times$ 10^{-4} s^{-1} for the native and denatured His resonances, respectively, with 6.0 M GdmCl.

this experiment at varying concentrations of GdmCl. We found that the fraction of His 15 unfolding in the burst phase depends on denaturant concentration, increasing from 0.06 in 4.5 M GdmCl to 0.46 in 8.0 M GdmCl (Figure 7).

GdmCl is a salt. Could the His 15 unfolding burst be caused by the high ionic strength of our 6.0 M GdmCl unfolding solution rather than by the action of GdmCl as a denaturant? To test this possibility, we recorded NMR

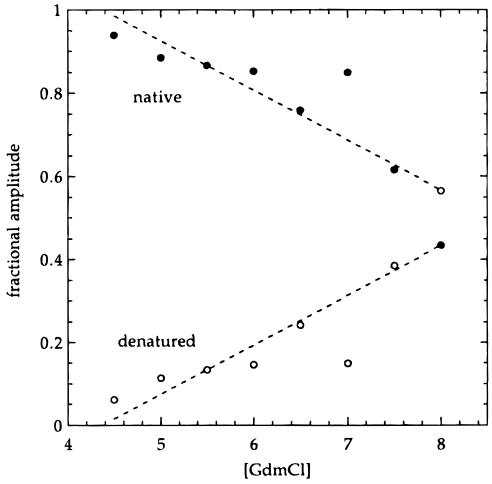

FIGURE 7: Variation in the burst amplitude of the denatured His 15 resonances (extrapolated from the single-exponential fit) versus GdmCl denaturation. Native His 15 resonance amplitudes are shown as filled circles; those of denatured His are shown as open symbols. The least-squares fits to lines are as follows: peak height = −13000[GdmCl] + 119000 $R = 0.94$ (native) and peak height = 3200[GdmCl] + −10000 $R = 0.73$ (denatured).

spectra of lysozyme in 1.0 M NaCl at pH* 4.0 at 10 and 25 °C. In these spectra, only the native His 15 resonance is seen, indicating that high ionic strength cannot locally unfold the His 15 region (data not shown). In contrast, an unfolding burst is seen when unfolding is caused by 8 M urea, a nonionic denaturant (data not shown). Interestingly, at urea unfolding conditions (pH* 3.33, 25.0 °C, and 8 M urea) where lysozyme's stability is similar to our standard unfolding conditions (6.0 M GdmCl at pH* 4.0 and 10 °C), the fractional amplitude of burst unfolding is 0.29, which is very similar to that seen in GdmCl (0.30). When lysozyme is unfolded in a combination of 8 M urea and 1.8 M NaCl at pH* 3.33 and 25.0 °C, unfolding is incomplete and the unfolding rate decreases by a factor of 2.5 as compared to that when NaCl is absent (data not shown). This suggests that the ionic nature of GdmCl probably acts to stabilize lysozyme at low pH* rather than to unfold it partially.

How fast is the early His unfolding phase? To quantitate better the kinetics of this rapid burst phase, unfolding was initiated by injecting denaturant directly into the NMR tube containing native protein solution within the NMR probe. This reduced the experimental dead time to about 10 s, so the first spectrum could be recorded after only about 25 s. A sharp unfolded His 15 resonance is still observed in the first spectrum of this experiment (data not shown). We tried to measure directly exchange between native and burst-unfolded His 15 by saturation-transfer NMR. We saturated the burst-unfolded resonance and looked for a decrease in the amplitude of the native resonance. No such decrease was seen. It is possible that exchange between native and burst-unfolded His 15 occurs outside the narrow time window (0.5−12 ms) which would have been detected by this approach. Taken together, these data indicate that the burst phase occurs on a time frame of tens of milliseconds to seconds.

DISCUSSION

Circular Dichroism. Far-UV circular dichroism is a sensitive probe of secondary structure, particularly helical structure, in proteins. For the unfolding of lysozyme at pH* 4 and 10 °C in 6.0 M GdmCl, 100 mM NaCl, and 50 mM NaAc, we observed a single kinetic phase containing all the amplitude for the native to denatured transition by CD at 222 nm. These data indicate that the helical structure of lysozyme unfolds in a single slow phase, with a kinetic lifetime of 3300 s. In the case of lysozyme, the packing of cystine and aromatic residues makes a substantial contribution to the native far-UV CD spectrum, particularly between 225 and 230 nm (Chaffotte *et al.*, 1992; Hider *et al.*, 1988; Kosen *et al.*, 1981). Lysozyme unfolding, monitored by CD at 227 nm, is also monophasic and contains the full amplitude of the equilibrium unfolding reaction, suggesting that lysozyme cystine and aromatic residues unfold in the same, slow kinetic step as the helical structure. Moreover, spectra of lysozyme recorded during unfolding can be successfully modeled by weighted sums of the native and denatured spectra, which argues that these structural features unfold cooperatively.

UV Absorbance. Changes in protein UV absorbance are generally attributed to changes in solvent exposure, as the interaction of solvent with the energy levels of a chromophore alters the energy levels and hence the wavelengths at which light is absorbed in the transition (Cantor & Schimmel, 1989). Segawa and Sugihara (1984) assigned the UV absorbance difference at 301 nm for lysozyme unfolding to Trp 108, as this absorbance difference disappears when Trp 108 is esterified. Here, lysozyme unfolding monitored by UV absorbance at 301 nm reveals a single, slow transition, indicating that the absorbing chromophores become solvent exposed only in the last step of unfolding, coincident with the loss of secondary structure. This is consistent with the unfolding behavior of Trp 108 monitored by Trp indole nitrogen proton exchange.

Fluorescence and Trp Indole Protons. Unfolding of Trp residues, monitored by fluorescence, reveals a major kinetic phase with a slow, major phase and a minor (10%) faster phase. The rate of the slow phase observed by fluorescence is slightly faster (relaxation time of 2400 s) than the rate of the major slow phase observed by other techniques. As the observed unfolding kinetics do not account for the entire equilibrium unfolding amplitude, there may be a second minor kinetic phase, which is faster and unobserved. On the whole, these data indicate, however, that the Trp residues and their surroundings, the hydrophobic core of the α-domain, unfold in a single, slow step roughly coincident with the unfolding of helical secondary structure detected by CD. In future work, it may prove worthwhile to study in detail the factors affecting the rate of the slow-unfolding phase monitored by Trp fluorescence, as compared with other spectroscopies. Possibly, the faster rate of the major unfolding phase observed by fluorescence results from motion of the Trp residues, of the kind hypothesized in the dry molten globule model of an unfolding intermediate, but is too slow to detect by NMR methods.

The contribution of individual lysozyme Trp residues to the fluorescence emission spectrum has been studied in detail by chemical modification and fluorescent lifetime measurement studies (Imoto *et al.*, 1971; Formoso & Forster, 1975). Their studies found that Trp 62 and Trp 108 contribute most of the fluorescence emission of native lysozyme. Other Trp residues make small contributions to the fluorescence of folded lysozyme; these residues all lie near cystine or methionine sulfurs, which presumably act as efficient quenchers. As the fast minor phase observed in this work is an increase in fluorescence, it may result from a partial

loosening of tight packing around one of the buried Trp residues, which reduces quenching efficiency. By recording NMR spectra of the Trp residues during unfolding, we found, however, that the buried indole nitrogen protons of Trp 108, 111, and 123 retain their native chemical shift values and peak widths prior to unfolding. Also, exchange of these indole protons shows that these residues become exposed to solvent cooperatively at a rate that matches the loss of helical structure observed by CD. If the Trp residues, or their surrounding residues, were to become mobile prior to becoming solvent exposed, their magnetic environments would be altered and the NMR spectra would reveal broadened lines, chemical shift changes, or both, as was seen for ribonuclease A and dihydrofolate reductase. These changes are not seen for the Trp residues of lysozyme. This experiment, therefore, is good evidence that the lysozyme core does not form a dry molten globule during unfolding. The fast, minor unfolding phase observed by fluorescence is better explained by Trp 63 unfolding and exchange, as this fluoresence phase has the same rate as the Trp 63 indole proton exchange. As Trp 63 and Trp 62 are exposed and located at the active site in a hinge region between the α- and β-domains, they are not representative of the hydrophobic core and may be more susceptible to unfolding than the other Trp residues.

Experiments To Quantitate Burst Unfolding. Aqueous solutions with a high ionic strength, such as 6.00 M GdmCl, can weaken the intensities of NMR lines (H. Lu and C. Dobson, personal communication). When lysozyme was titrated with GdmCl, we observed a strong decrease in the intensity of the His 15 native resonance with increasing GdmCl concentrations (data not shown).[2] For this reason, we found it difficult to quantitate possible unfolding occurring in the 3 min dead time of the NMR experiment by comparing intensities of the native lysozyme resonances in buffer to the same native resonances in spectra recorded early in the unfolding reaction in GdmCl solution. Instead, three approaches were used to detect rapid, early unfolding. Two experiments utilized the lysozyme Trp indole nitrogen protons: monitoring their behavior during unfolding in H_2O, rather than in D_2O, and a pulse/quench experiment to label any Trp that unfolds and becomes solvent exposed in the unfolding dead time.

By following the unfolding of lysozyme's Trp residues in H_2O, we detected a small unfolding burst as an excess in the denatured resonance area, when extrapolated back to the start of unfolding. This unfolding burst is small; it is equivalent in amplitude to only 0.6 Trp residue. The likely source of this unfolding burst is Trp 62, Trp 63, or both, whose combined native peak area at the start of unfolding is smaller than expected. Another possible but less likely source for the NMR-detected unfolding burst and the minor, rapid fluorescence phase is Trp 28. The unfolding behavior of Trp 28 could not be determined, as its NMR resonance is obscured by overlapping amide proton resonances in the 1D ^{1}H-NMR spectrum. As Trp 28 is completely buried, and its indole nitrogen proton is hydrogen bonded, we speculate that the unfolding behavior of Trp 28 resembles that of the buried Trp residues.

[2] In the experiments of Kiefhaber *et al.* (1995) on RNase A, the effect of GdmCl on the intensity of the native Val 63 resonance line was found to saturate at 0.5 M GdmCl (T. Kiefhaber, personal communication).

The pulse/exchange experiment was unable to detect any unfolding of the buried Trp residues (123, 111, and 108) in the first minutes of unfolding. Taken together with unfolding experiments monitored in real time (after a 3 min dead time), which show that Trp indole nitrogen protons resonate at their native chemical shift values prior to slow, global unfolding, this is excellent evidence that the hydrophobic core is dry and retains its native-like tight packing prior to the slow, cooperative step of unfolding.

Histidine ^{1}H-NMR Spectroscopy. The third approach was to measure unfolding directly by following the appearance of the unfolded His 15 CϵH resonance, which gives an isolated resonance in the downfield 1D NMR spectrum, after deuteration of the amide protons. A major fraction of His 15 unfolds in a slow kinetic phase. The rate of this slow-unfolding phase matches that of the unfolding of buried Trp residues and the loss of structure detected by CD. However, a large minority of the His 15 amplitude (30% in our standard conditions) undergoes a rapid unfolding reaction, and the amplitude of this phase increases with increasing GdmCl concentrations. If one assumes that, early during folding, native lysozyme and the burst phase intermediate detected by NMR are in a two-state equilibrium (which can only be approximately true), an estimate of 450 cal mol^{-1} M^{-1} can be calculated for the *m* value of the formation of the intermediate. This corresponds to roughly 23% of the *m* value observed for global unfolding (Figure 2A).

Can the environment of His 15 explain its propensity to show rapid partial unfolding? His 15 lies at the end of the first helix in lysozyme and is about 20% solvent exposed. His 15 contacts Arg 14, Asp 87, and Thr 89. An imidazole nitrogen proton of His 15 is hydrogen bonded to the Thr 89 side chain (Beddell *et al.*, 1975). The breaking of this hydrogen bond and the loss of local tight packing are probably the local structural changes detected by NMR. It is plausible that charge repulsion between Arg 14 and His 15, whose side chain is ionized at pH 4, predisposes this region for local unfolding. Nevertheless, quantitatively similar dead time unfolding bursts are seen in GdmCl, in urea plus 1.8 M NaCl (solutions where the high ionic strength ought to screen electrostatic interactions), as well as in urea without salt. This suggests that the predisposition of this region to unfold is not electrostatic in nature. In known avian lysozyme sequences, hen and quail egg white lysozymes have a histidine residue at position 15; however, but four other species have a Leu at position 15, and His is replaced by Met in a fifth species (Jolles *et al.*, 1979; Araki *et al.*, 1990). Interestingly, the Leu 15 variant adopts a completely different conformation than His; by rotating, the Leu side chain becomes deeply buried in the hydrophobic core (Banyard *et al.*, 1974). In the context of hen lysozyme, the Leu variant is more stable than the His variant by about 1 kcal/mol despite the loss of the hydrogen bond interaction with Thr 89 (Shih & Kirsch, 1995). These inferences suggest that the local structure about His 15 is not very stable and may be susceptible to early unfolding.

Our preparation of lysozyme samples for NMR study involved repeated heating and freeze-drying steps to deuterate the protein's amide groups. Might this harsh treatment lead to the formation of irreversibly unfolded protein that accounts for the burst phase in unfolding? Several lines of evidence suggest that this is not occurring. First, no evidence for dead time-unfolded material was seen by other spectroscopic techniques such as CD, when unfolding was performed with protein samples identical to those used in the NMR experi-

ments. An NMR spectrum of lysozyme dissolved in native buffer showed no evidence of unfolded histidine, or any other residue. Aggregation was observed only when lysozyme is highly concentrated, at 30 mg/mL or more, in 1.0−2.0 M GdmCl or >2 M NaCl. Moreover, similar burst phase amplitudes were seen whether unfolding was initiated from dry lyophilized protein or protein that had been preincubated in buffer, to allow any structure which may have unfolded upon lyophilization to refold. In addition, the equilibrium unfolding of lysozyme was measured by NMR and by far-UV CD. The same preparation of protein gave identical transition curves and showed no evidence for unfolded His below 2.75 M GdmCl. Increasing the concentration of the denaturant, GdmCl, decreases the intensities of native NMR resonance lines (data not shown; H. Lu and C. M. Dobson, personal communication). Since high concentrations of GdmCl can only decrease the intensity, this effect cannot account for the early appearance of the unfolded histidine 15 Cϵ1H resonance. Finally, the fraction of the His 15 resonance that unfolds rapidly (the burst amplitude) increases with increasing GdmCl concentrations. If lysozyme were being damaged in preparation, the burst amplitude would not depend on the final GdmCl concentration.

Comparisons with Other Studies. It is notable that most of the probes used here to follow lysozyme unfolding are found in the α-domain of lysozyme. His 15 and Trp 28, 108, 111, and 123 are located in the α-domain, while Trp 62 and 63 lie at the active site hinge region between the α- and β-domains. CD is most sensitive to the helical structure, cystine, and aromatic residues, most of which lie in the α-domain. As some evidence has suggested that the β-domain might unfold before the α-domain, it would be interesting to characterize the unfolding behavior of this region by specific ^{13}C labeling and NMR spectroscopy.

How do these results for lysozyme unfolding compare to those recently observed for RNase A and dihydrofolate reductase? In RNase A, valine 63 loses most of its native ^{1}H-NMR resonance line in the dead time (1 min) of unfolding, and the amount of native resonance lost increases with the concentration of GdmCl (Kiefhaber *et al.*, 1995). This unfolding behavior has a larger dead time amplitude but is otherwise similar in character to that exhibited by histidine 15 of lysozyme, as monitored by ^{1}H-NMR. Like His 15 of lysozyme, the Val 63 of RNase A is partially exposed and not deeply packed within the hydrophobic core. From work with RNase A and lysozyme, we begin to see a general picture of noncore residues becoming mobile early in protein unfolding. In the DHFR unfolding intermediate, disruption of the hydrophobic core is thorough and cooperative, as shown by the fast and complete disappearance of all five ^{19}F-labeled Trp NMR native state lines (Hoeltzli & Frieden, 1995). The lysozyme and DHFR results are not necessarily contradictory. Unfolding of DHFR was followed by ^{19}F-NMR spectroscopy, a very sensitive probe of environmental changes. During unfolding, the lysozyme core structure might undergo a slight loosening that is undetected by ^{1}H-NMR spectroscopy but would be detected by ^{19}F-NMR. DHFR was unfolded, moreover, without its large methotrexate cofactor and dihydrofolate and NADPH substrates. Lacking the tertiary contacts provided by these molecules, the hydrophobic core of DHFR may be especially susceptible to unfolding. In the lysozyme active site, Trp 62 and Trp 63 side chains are exposed and mobile in the absence of substrate.

Thus, a search for a "dry molten globule" intermediate in the unfolding of lysoyzme has produced results different from the ones found earlier for ribonuclease A and dihydrofolate reductase. More work is needed to find out if these different results can be fitted into a consistent view of the role of such intermediates in protein unfolding.

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NOTE ADDED IN PROOF

A similar fast fluorescence-detected unfolding phase has been observed by T. Kiefhaber, who has assigned it to the binding of lysozyme to the cuvette wall (T. Kiefhaber, unpublished, private communication).

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