Partially Folded States of Equine Lysozyme. Structural Characterization and Significance for Protein Folding†

Herman Van Dael,*,‡ Petra Haezebrouck,‡ Ludmilla Morozova,‡,§ Christopher Arico-Muendel,∥,⊥ and Christopher M. Dobson∥

Interdisciplinary Research Centre, K.U. Leuven Campus Kortrijk, B-8500 Kortrijk, Belgium, and Oxford Centre for Molecular Sciences and Inorganic Chemistry Laboratory, University of Oxford, South Parks Road, Oxford OX1 3QR, England

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ABSTRACT: Despite their homologous structure, *c*-type lysozymes and α-lactalbumins have been found to differ profoundly in their unfolding behavior, in that the α-lactalbumins readily enter a partially unfolded collapsed state (the "molten globule"), whereas lysozymes unfold cooperatively to a highly unfolded state. The calcium-binding property of lysozyme from equine milk provides an evolutionary link between the two families of proteins. We demonstrate here that equine lysozyme undergoes a two-stage unfolding transition upon heating or in the presence of guanidine hydrochloride that is highly dependent on the state of calcium binding. Differential scanning calorimetry shows the two transitions to be particularly well resolved in the calcium-free protein, where the first transition occurs with a midpoint at 44 °C at pH 4.5 or in 0.8 M GdnHCl at pH 7.5, 25 °C, and the second occurs near 70 °C at pH 4.5 or in 3.7 M GdnHCl at pH 7.5, 25 °C. In the presence of calcium, the first transition takes place with a midpoint of 55 °C or in excess of 2.5 M GdnHCl, but the parameters for the second transition remain unchanged. Fluorescence emission and UV difference absorption spectroscopy suggest that the first transition generates an intermediate state in which sequestration of some aromatic side chains from solvent has occurred whereas the second represents denaturation to a highly unfolded state. CD and ^{1}H NMR results indicate that the intermediate state possesses extensive secondary and tertiary structure, although the latter is substantially disordered. Although the equine lysozyme unfolding intermediate resembles the partially denatured collapsed state identified for α-lactalbumins, the magnitude of its near-UV CD signal and the large enthalpy change associated with the second transition suggest that its structure is significantly more highly organised.

Partially denatured states of small, globular proteins may be of major importance in helping to elucidate protein folding mechanisms (Dobson, 1992). The folding properties of *c*-type lysozymes and α-lactalbumins from a number of sources have been studied in detail, and have shown that lysozymes unfold according to the classical two-state model for protein denaturation, whereas α-lactalbumins readily enter a compact, partially unfolded state termed the "molten globule" which is characterized by a high degree of secondary structure and a lack of tertiary structure (Tanford et al., 1973; Kuwajima, 1989). This difference is particularly remarkable in light of the high sequence and structural homology of the two proteins. The cause of these differences remains elusive, though the observation (Buck et al., 1993) that partial denaturation of hen lysozyme may be induced in trifluoroethanol suggests that lysozymes may be unfolded noncooperatively under certain conditions. An alternative approach to the issue may be sought in the study of lysozymes that are more closely related to α-lactalbumins as a result of their position in the evolutionary history of the two protein families.

In contrast to most *c*-type lysozymes, equine milk lysozyme binds Ca^{2+} (Nitta et al., 1987, 1988; Desmet et al., 1989). Recent crystallographic studies of equine lysozyme at 2.5-Å resolution have demonstrated that the conformation of the Ca^{2+}-binding loop is similar to that found in all species of α-lactalbumin (Tsuge et al., 1992). For the latter protein, it has been shown (Stuart et al., 1986) that the Ca^{2+}-binding site is composed of three Asp side chains and two main-chain carbonyl groups. This site is entirely conserved in equine lysozyme and is composed of Asp-85, -90, and -91, Lys-82, and Asn 87 (residue numbering is based on that for equine lysozyme). The overall structure of equine lysozyme is substantially the same as those of the non-Ca^{2+}-binding lysozymes and α-lactalbumins (Tsuge et al., 1992). A deep cleft containing the active site divides the lysozyme molecule into two domains: domain 1 is a β-sheet-rich structure while domain 2 contains the α-helices and both the N- and C-terminal segments.

The effects of Ca^{2+} binding on the conformation and on the stability of equine lysozyme, however, seem to differ from those observed in α-lactalbumins. The circular dichroism (CD)[1] spectra of apo and holo forms of equine lysozyme at 25 °C show only subtle differences upon Ca^{2+} binding (Desmet et al., 1989). In contrast, the CD spectrum of α-lactalbumin

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* To whom correspondence should be addressed.

‡ University of Leuven Campus Kortrijk.

§ On leave from the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region. Present address: Max Planck Institute für Biochemie, Martinsried, Germany.

∥ University of Oxford.

⊥ Present address: Department of Chemistry, Brandeis University, Waltham, MA 02254.

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[1] Abbreviations: CD, circular dichroism; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid; GdnHCl, guanidine hydrochloride; NMR, nuclear magnetic resonance.

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shows a dramatic decrease in the near-UV region, corresponding to the appearance of the molten globule state (Segawa & Sugai, 1983). From two-dimensional ^{1}H NMR measurements, Tsuge et al. (1991) also concluded that the conformational changes between the holo and apo structures of equine lysozyme were different from those of α-lactalbumin, although they accepted that, like in α-lactalbumin, the binding process provokes a global conformational change that can be sensed throughout the whole protein. From fluorescence measurements on mixtures of bis-ANS and equine lysozyme and from Ca^{2+}-dependent hydrophobic interaction chromatography of equine lysozyme, it was demonstrated (Haezebrouck et al., 1992) that Ca^{2+} binding induces a conformational change upon which hydrophobic regions in the protein become less accessible. This effect was also observed in α-lactalbumin (Haezebrouck et al., 1992; Musci & Berliner, 1985). As phenyl-Sepharose binds apo α-lactalbumin in preference to apo equine lysozyme, however, equine lysozyme has a less hydrophobic character than α-lactalbumin (Haezebrouck et al., 1992).

Our earlier study of the thermal folding behavior of equine lysozyme suggested a two-stage mechanism that was particularly evident for the apo protein (Morozova et al., 1991). An initial blue shift in the fluorescence emission spectrum and a small change in the near-UV CD data suggested that the first transition involved the reorganization and burial of some aromatic side chains. A subsequent red shift in the fluorescence spectrum, accompanied by large decreases in the near- and far-UV CD intensity, was attributed to the general loss of secondary and tertiary structure.

In the present paper, we offer further evidence for two-stage unfolding, in response to both heating and the addition of chemical denaturant, and describe the characterization of the intermediate state resulting from the first unfolding transition. To this end, we employ a number of spectroscopic probes, including CD, fluorescence, and difference absorption techniques, and particularly NMR spectroscopy. The optical methods are highly sensitive to changes in both secondary and tertiary structure and can supply some information regarding the types of residues undergoing transitions. NMR, on the other hand, is an extremely sensitive monitor of conformational properties and conformational changes of individual residues in proteins. The structures of native and denatured states, and the folding behavior of a number of lysozymes and α-lactalbumins, have been investigated by NMR (Dobson & Evans, 1984; Baum et al., 1989; Miranker et al., 1991; Radford et al., 1992; Alexandrescu et al., 1993; Smith et al., 1993), and the detailed 2D analysis of the native state of equine lysozyme is in progress. The spectroscopic data are, in addition, shown to agree well with DSC studies that reveal the two-stage nature of the apo enzyme transition explicitly. The intermediate that forms in the apo enzyme is, we believe, a particularly interesting example of a non-native, partially folded state of a protein. It has many characteristics of the classic "molten globule" states of α-lactalbumins but appears to have somewhat more highly ordered tertiary interactions and, most importantly, a significant enthalpic barrier to more complete unfolding. Its further study should provide valuable insights into the factors stabilizing structural features of proteins and into the folding mechanisms of lysozymes and lactalbumins, as well as other small globular proteins.

MATERIALS AND METHODS

Equine lysozyme was isolated from Clydesdale horse milk, purified, and decalcified as described extensively in a previous paper (Haezebrouck et al., 1992). The residual Ca^{2+} content in the apo protein, checked by atomic absorption, was always less than 0.03 mol/mol of protein. Protein concentration was determined by absorbance measurement at 280 nm using an extinction coefficient of $E_{1\%} = 23.5$. Unless indicated otherwise, all experiments were done with protein concentrations between 20 and 30 μM in Tris buffer at pH 7.5 and $T = 25$ °C. Extremely pure GdnHCl was purchased from U.S. Biochemicals.

The circular dichroism experiments were carried out on a Jasco J-600A spectropolarimeter using cuvettes of 1- or 0.1-cm path length. Base-line normalization was done at 250 nm and at 320 nm, respectively. Results were expressed as the mean residue ellipticity, $[\theta]$ (deg·cm^2/dmol), using 112.7 as the mean residue weight for equine lysozyme.

Fluorescence and absorption spectroscopies were performed on an Aminco SPF-500 spectrofluorometer and on a Beckman DU-70 spectrophotometer, respectively. The ratio of fluorescence intensities at fixed wavelengths on the wings of the spectrum (I_{370}/I_{330}) was used as a measure for spectrum position (Morozova et al., 1991). Difference absorption spectra at each temperature were obtained by subtraction of the spectrum at this particular temperature from the spectrum at low temperature, usually at 10 °C.

Exchangeable protons of samples for NMR were fully deuterated by heating in D_2O at 65–70 °C for 10 min. The pH was adjusted with small quantities of NaOD and DCl, and readings were not corrected for isotope effects. Guanidine hydrochloride was fully deuterated by repeated lyophilization from D_2O. Samples of apo enzyme were handled with glassware that had been rinsed with HCl and EDTA; samples of Ca^{2+}-bound enzyme contained in addition 1 mM $CaCl_2$. Final sample concentrations in NMR experiments ranged from 0.6 mM (GdnHCl experiments, at pH 7.5, 25 °C, 80 mM NaCl + 20 mM Tris) to 1.0 mM (variable-temperature experiments at pH 4.5).

One-dimensional ^{1}H NMR experiments were obtained on a Bruker AM-600 instrument of the Oxford Centre for Molecular Sciences. Relaxation delays of 0.8–0.9 s were left between acquisitions, during which the residual HOD signal was attenuated by presaturation. In all cases, the sweep width was 13.0 ppm, and the digital resolution 0.95 Hz/point. One-half hertz line broadening was routinely applied during transformation. Dioxane was employed as an internal chemical shift reference at 3.74 ppm. Variable-temperature studies of apo equine lysozyme were conducted at 2 °C intervals between 28 and 52 °C, and at 5 °C intervals between 55 and 82 °C, whereas for the holo protein, spectra were recorded at 2 °C intervals between 46 and 64 °C and at 5 °C intervals between 31–42 and 68–77 °C. In both cases, the instrument temperature readout was calibrated with ethylene glycol.

Differential scanning calorimetry was carried out at 1 K per minute heating rates on a DASM-4 microcalorimeter in the Institute of Theoretical and Experimental Biophysics, Pushchino (Russia). The operational volume of the platinum measuring cell was 0.5 mL and the protein concentration 2.2 mg/mL. The stability of the base line allowed the partial heat capacity to be determined with an error of not more than 2%. As the DSC traces are asymmetric, the temperatures of the various maxima do not coincide exactly with the midpoint of the phase transition or the conformational change. Experimental points were therefore fitted with a combination of Gaussian functions in order to obtain the thermal characteristics (transition temperature, band width, and peak height) of the separate processes.

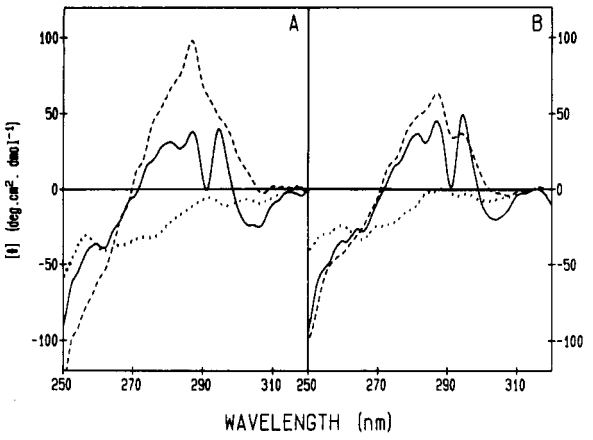

FIGURE 1: Near-UV CD spectra of equine lysozyme at 25 °C with different concentrations of GdnHCl. (A) Apo form in 20 mM Tris/80 mM NaCl, pH 7.5, with (—) 0, (- - -) 2.01, and (···) 6.46 M GdnHCl. (B) Ca^{2+} form in 20 mM Tris/80 mM NaCl/10 mM Ca^{2+}, pH 7.5, with (—) 0, (- - -) 3.15, and (···) 6.62 M GdnHCl. The buffer spectrum is subtracted.

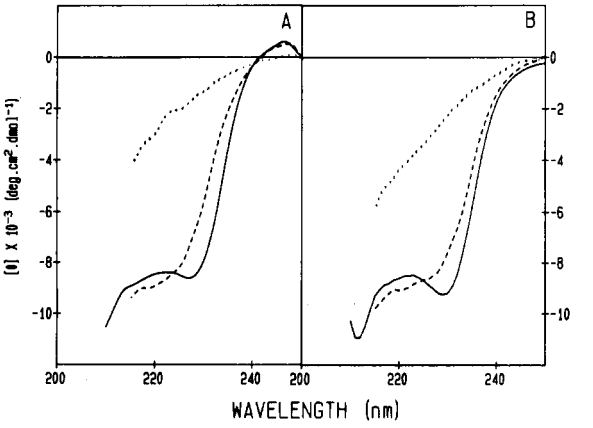

FIGURE 2: Far-UV CD spectra of equine lysozyme at 25 °C with different concentrations of GdnHCl. (A) Apo form in 20 mM Tris/80 mM NaCl, pH 7.5, with (—) 0, (- - -) 1.97, and (···) 5.53 M GdnHCl. (B) Ca^{2+} form in 20 mM Tris/80 mM NaCl, pH 7.5, with (—) 0, (- - -) 2.74, and (···) 4.51 M GdnHCl.

RESULTS

Circular Dichroism Spectra and Chemical Unfolding. The CD spectra of apo (A) and Ca^{2+} equine lysozymes (B) are depicted in Figure 1 for the near-UV region and in Figure 2 for the far-UV region. The near-UV spectrum of the native protein shows three maxima at 282, 287, and 294 nm, in agreement with our previous results at the same pH (Desmet et al., 1989). They also agree with the spectra at pH 4.5 that we reported previously (Morozova et al., 1991), although the fine structure that was detected between 275 and 285 nm disappears at neutral pH. The addition of GdnHCl (2 M) to the apo form causes such an enhancement of the 287-nm peak that the other two maxima appear only as shoulders on the central peak. A high concentration (6 M) of denaturant, however, provokes a complete loss of signal characteristic of tertiary structure. In the Ca^{2+} form, the increase of the 287-nm maximum following addition of moderate concentrations of GdnHCl (3 M in Figure 1) occurs to a much smaller degree. This difference in the behavior of the apo and holo proteins is not found in the far-UV region (Figure 2) where GdnHCl influences the spectra of both forms in a similar way. The addition of 2–3 M GdnHCl to the apo form and 2.5–3 M GdnHCl to the calcium form causes a slight enhancement of the CD signal in the region 218–223 nm, whereas the local

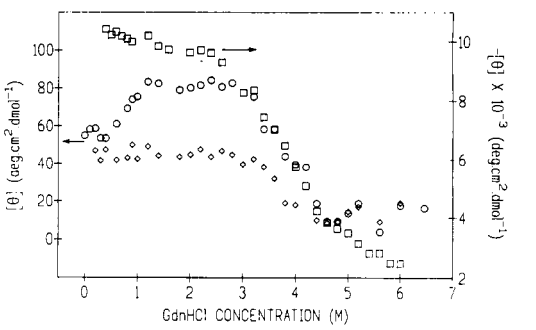

FIGURE 3: Chemical denaturation of apo equine lysozyme at 25 °C in 20 mM Tris/80 mM NaCl, pH 7.5, followed by CD at 287 (○), 294 (◇), and 222 nm (□).

minimum at 228 nm disappears (Figure 2). The secondary structure that is conserved for the most part at moderate GdnHCl concentration is strongly reduced at high denaturant concentration.

In Figure 3, the chemical unfolding of the apo protein upon addition of GdnHCl is shown in the near-UV (left axis) and in the far-UV (right axis) regions. In both spectral regions, a cooperative unfolding transition takes place between 2.7 and 5 M GdnHCl with a common midpoint, $C_{1/2}$, at 3.7 M. Upon comparing this result with the GdnHCl unfolding of bovine apo α-lactalbumin (Ikeguchi et al., 1986), two remarkable features stand out. First, for apo α-lactalbumin, the unfolding at 270 nm ($C_{1/2}$ = 1.4 M) markedly precedes the unfolding at 222 nm ($C_{1/2}$ = 2.3 M). Equine lysozyme obviously does not show such behavior. Second, in equine lysozyme, the main transition takes place at significantly higher denaturant concentration ($C_{1/2}$ = 3.7 M), indicating a higher intrinsic stability. This is in agreement with our earlier studies (Morozova et al., 1991), in which we demonstrated that the thermal stability of equine lysozyme, although distinctly lower than that of hen and human lysozymes, markedly exceeds that of α-lactalbumins.

The most striking feature from Figure 3, nevertheless, is the net increase of the ellipticity at 287 nm in the concentration range 0.5–1.2 M GdnHCl. Subsequently, the ellipticity remains practically constant until the start of the main transition near 3 M GdnHCl. This phenomenon, also detectable from the wavelength scans of Figure 1, indicates that the environment of the tryptophan group(s) responsible for the 287-nm signal is altered at low denaturant concentrations. As the CD signal of a specific group increases when the motion of this group is restricted by a more structured environment, this first transition could be related to the transfer of some exposed tryptophan group(s) to a more hydrophobic location. A similar enhancement of the ellipticity was found in the thermal unfolding curves recorded at 289 nm (Morozova et al., 1991). The fact that no such ellipticity increase is observed in the spectra recorded at 294 nm indicates that not all the tryptophan groups are affected in the same way.

Figure 4 shows the chemical denaturation curves in both spectral regions for the Ca^{2+}-loaded protein. From the ellipticity measurements in the far-UV region, information on changes in secondary structure can be derived. Up to 2.5 M GdnHCl, the ellipticity at 222 nm remains relatively constant in contrast to the small decrease in ellipticity observed in the apo protein over that concentration range (Figure 3). The second transition, however, has its midpoint at a GdnHCl concentration of 3.7 M, exactly the same value as in the case of the apo protein. Thus, the binding of Ca^{2+} has no direct effect on the residual denaturation of equine lysozyme in GdnHCl, as was also concluded in our thermal unfolding study

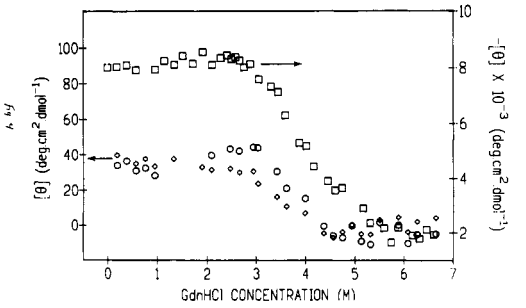

FIGURE 4: Chemical denaturation of Ca^{2+} equine lysozyme at 25 °C in 20 mM Tris/80 mM NaCl, pH 7.5, followed by CD at 287 (○), 294 (◇), and 222 nm (□).

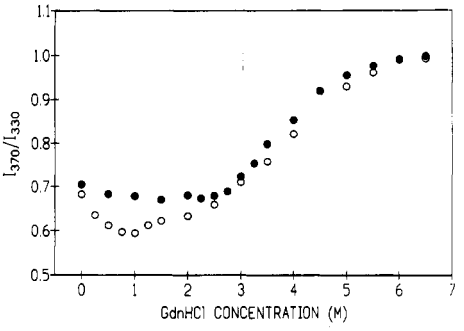

FIGURE 5: Chemical denaturation of equine lysozyme measured by fluorescence spectroscopy: (○) apo form and (●) Ca^{2+} form in 20 mM Tris/80 mM NaCl, pH 7.5.

(Morozova et al., 1991). In the near-UV region (Figure 4, left axis), the main transition has the same width and midpoint as discussed above for the far-UV region. Again, Ca^{2+} binding does not induce an obvious change in the overall stability of equine lysozyme. Measured at 287 nm, a pretransitional increase of ellipticity is observable but much less pronounced than in the case of the Ca^{2+}-free protein (Figure 3). The conformational effects which give rise to the first transition are clearly dependent on Ca^{2+} concentration.

Fluorescence Spectroscopy. The study of the tryptophan fluorescence emission spectrum provides additional information about the nature of conformational changes induced by Ca^{2+} binding. Specifically, a good indication of spectral changes may be found in the intensity ratio of the fluorescence signal at both sides of the spectral maximum. An increase of that ratio, indicating a red shift of the spectrum, corresponds to a transfer of some tryptophan residues from the hydrophobic interior to a more solvent-exposed position in the protein (Permyakov et al., 1981). Figure 5 shows I_{370}/I_{330} for apo- and Ca^{2+}-bound equine lysozyme as a function of GdnHCl concentration. For both samples, the midpoint of the main transition lies at 3.7 M, in accordance with the CD results from the previous section. For the apo form at low GdnHCl concentration (about 1 M), a decrease in the intensity ratio is observed, as would be expected for a transfer of tryptophan groups to buried positions.

Difference Absorption Spectroscopy. Thermally induced perturbations of a protein, particularly involving tryptophan and tyrosine residues, can be identified by means of difference absorption spectra (Nicola & Leach, 1976). As can be seen in Figure 6, the thermal transition of equine lysozyme at pH 4.5 gives rise to a difference absorption spectrum with maxima at 282 and 291 nm. As in hen egg white lysozyme, where they appear at 286 and 294 nm (Bello, 1970; Leach & Smith, 1972), these peaks are almost certainly due to changes in the environment of tryptophan residues. From this figure, we also detect the influence of Na^+ and Ca^{2+} ions on the thermal

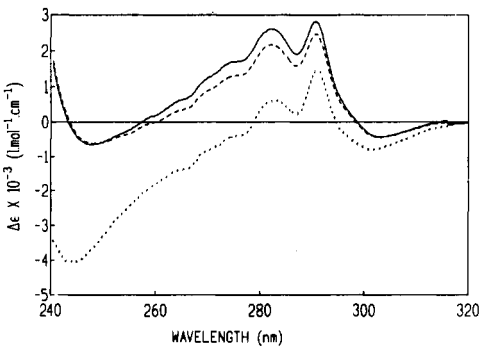

FIGURE 6: Difference spectra of equine lysozyme at 90 °C, pH 4.5, (···) in 10 mM ammonium acetate/2 mM EDTA, (- - -) in 10 mM sodium acetate/90 mM NaCl, and (—) in 10 mM sodium acetate, 90 mM NaCl, and 10 mM Ca^{2+}. Base temperature = 10 °C.

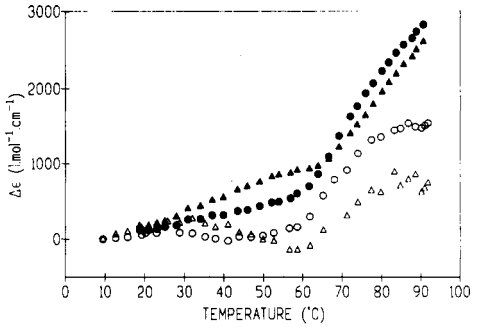

FIGURE 7: Thermal transition of equine lysozyme measured with differential absorption spectroscopy (pH 4.5) in 10 mM ammonium acetate/2 mM EDTA (open symbols) and in 10 mM sodium acetate, 90 mM NaCl, and 10 mM Ca^{2+} (filled symbols) at 282 nm (triangles) and 291 nm (circles).

unfolding characteristics of equine lysozyme.

Figure 7 shows the thermal denaturation curves of apo equine lysozyme and its Ca^{2+}-loaded counterpart at 282 and 291 nm. First of all, we note the main transition in the temperature range 60–90 °C, in which tryptophan groups become more exposed to the solvent (Donovan, 1973). As pointed out earlier (Morozova et al., 1991), Ca^{2+} ions do not shift the temperature interval in which this transition occurs. Furthermore, an obvious pretransitional effect can be observed. For the apo form, this effect is most pronounced when measured at 282 nm. The small linear increase of the signal between 10 and 30 °C is unlikely to be due to any conformational changes in the protein, but rather may be associated with a direct effect of temperature on the chromophore (Nicola & Leach, 1976). The decrease between 30 and 60 °C, however, may be explained by movement of tryptophan residues toward a more hydrophobic environment (Donovan, 1973). The addition of Ca^{2+} to the protein (Figure 7, full symbols) could obstruct such movement, so that the signal increases linearly until 55 °C. The nature of the first transition may be studied more unambiguously at pH 7.5, where it is shifted to a lower temperature and thus does not overlap with the main transition. Due to turbidity observed at high temperature, the data of Figure 8 are presented in a restricted temperature range where the transition remains completely reversible and association of the protein can be excluded. In contrast to the main transition that is unaffected by Na^+ or Ca^{2+} binding, the first transition is clearly influenced by metal binding to the protein. The decrease in the difference absorption signal starts at 20 °C for the apo sample (Figure 8A) and only at 30 °C and approximately 50 °C for the Na^+ (Figure 8B) and Ca^{2+} (Figure 8C) samples, respectively. We have recorded spectra at four different wavelengths: at 281

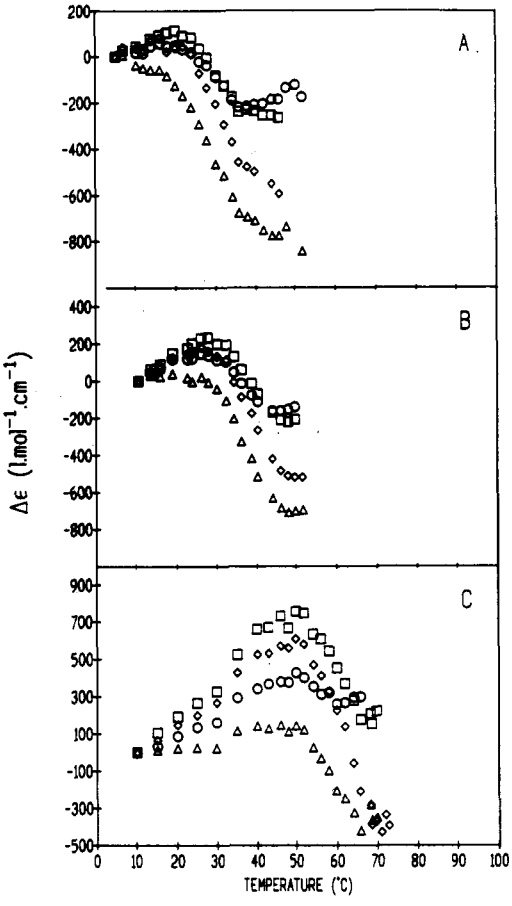

FIGURE 8: Thermal transition of equine lysozyme measured with differential absorption spectroscopy (pH 7.5) at 275 (◇), 281 (□), 287 (△), and 290 nm (○). (A) 10 mM Tris/2 mM EDTA; (B) 10 mM Tris, 2 mM EDTA, and 0.1 M NaCl; (C) 10 mM Tris/2 mM Ca^{2+}.

and 290 nm, the difference spectra are generated only by tryptophan groups, while at 276 and 285 nm tyrosine groups could also be involved (Nicola & Leach, 1976). The first transition, which again appears to involve the transfer of aromatic side chains to the protein interior, is thus strongly dependent on the state of metal ligation.

^1H NMR Studies. (A) Thermal Unfolding of Equine Lysozyme at pH 4.5. One-dimensional ^1H NMR spectra of apo- and Ca^{2+}-bound equine lysozyme at pH 4.5, 31 °C, are compared in Figure 9. The spectra closely resemble those of Tsuge et al. (1991) acquired at pH 7.5, and the high degree of resonance dispersion in the aliphatic and aromatic regions confirms that both forms of the enzyme are nativelike under these conditions. Lines in the spectrum of the apo enzyme are noticeably broadened, however, which may be due to conformational exchange processes occurring at rates near the NMR time scale ($k \sim 10^2$–10^3/s). Spectra of the apo protein acquired at 10- and 100-fold dilution display similar line widths and chemical shifts, suggesting that molecular association is not the cause of the broadening. Most of the well-dispersed resonances have been assigned to specific residues in the protein by 2D COSY and NOESY experiments (Arico-Muendel et al., unpublished results). Figure 9 shows that these residues are well distributed over the protein sequence, providing structural reporters throughout the molecule.

The thermal unfolding transitions were monitored with 1D NMR spectra acquired at 2 °C intervals in the transition regions and 5 °C intervals elsewhere. Heating the apo enzyme induces a major change in the NMR spectrum in the range

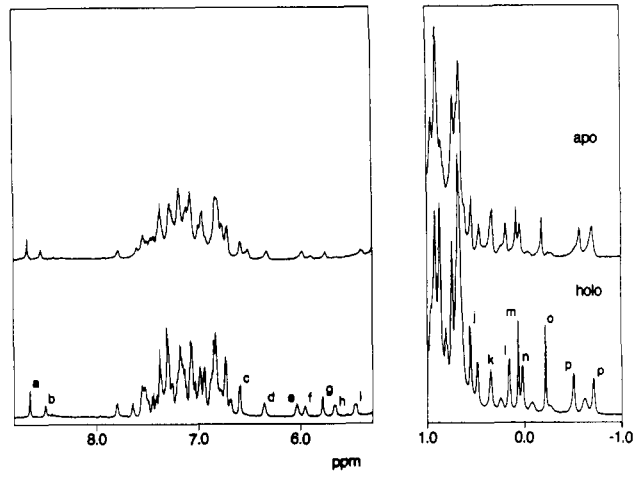

FIGURE 9: NMR spectra of apo (top) and holo (bottom) equine lysozymes at pH 4.5, 31 °C. Resonances identified in the spectrum of holo (=Ca^{2+}-bound) equine lysozyme are labeled: (a) H10; (b) H114; (c) Y34; (d) W108; (e) W28; (f) N60; (g) C65; (h) L84; (i) N39; (j) L129; (k) I88; (l) L56; (m) M17; (n) L8; (o) M105; (p) V98.

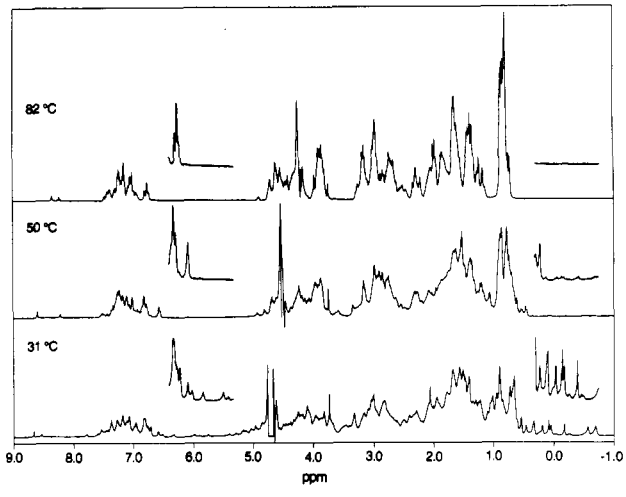

FIGURE 10: NMR spectra of apo equine lysozyme at pH 4.5, 82 °C (top), 50 °C (middle), and 31 °C (bottom). Vertical expansions correspond to the regions 0.56 to –0.54 ppm and 6.9–5.8 ppm.

33–47 °C, comparable with the range (30–50 °C) in which CD spectra show their first thermal transition (Morozova et al., 1991). The highly dispersed resonances corresponding to the native state show a decrease in intensity, concurrent with an intensity gain at chemical shifts characteristic of a denatured polypeptide (Figure 10). The existence of distinct sets of peaks indicates that the two protein conformational states are in slow exchange, with lifetimes of the order of seconds, suggestive of a cooperative unfolding process. Moreover, since all the native signals disappear simultaneously, the transition appears to involve the entire protein molecule. The spectrum at 50 °C, however, shows major differences from that of thermally denatured hen lysozyme (Evans et al., 1991), particularly in the degree of residual chemical shift dispersion. The aliphatic signal intensity remains upfield of 0.5 ppm, and in the aromatic region, a large, broadened resonance appears at 6.56 ppm. This peak is resolved at higher temperatures into a pair of doublets, which we have tentatively assigned as tyrosine resonances on the basis of their upfield shift and large splitting (Wüthrich, 1986). In addition, the ϵ_1 resonance of His-10 remains close to the expected random-coil shift under these conditions, whereas that of His-114 shifts upfield to 8.22 ppm. Broadened peaks and residual chemical shift dispersion are

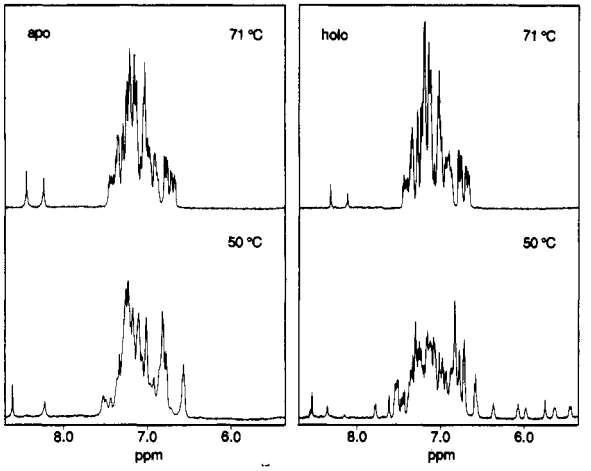

FIGURE 11: Aromatic regions of NMR spectra of apo (left) and holo (right) equine lysozymes at 50 and 71 °C, pH 4.5.

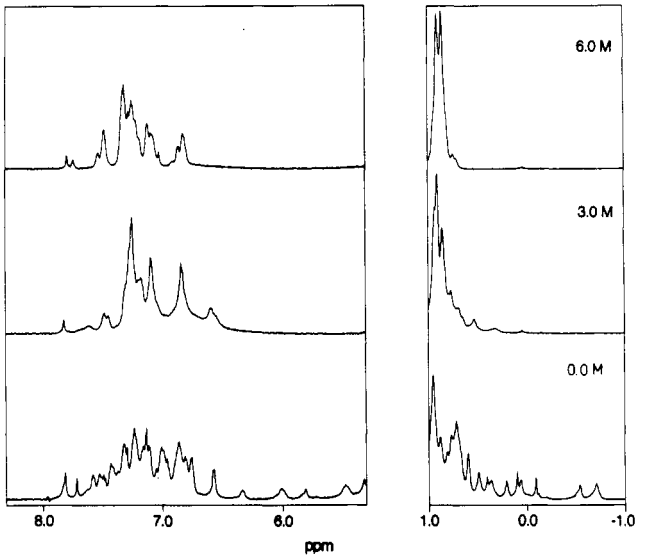

FIGURE 12: NMR spectra of apo equine lysozyme at pH 7.5, 25 °C, in 6 (top), 3 (middle), and 0 M GdnHCl (bottom).

also evident in the spectrum of thermally denatured α-lactalbumin (Baum et al., 1989), and are suggestive of conformational exchange processes occurring at intermediate rates, as well as some persistent structural interactions involving aromatic residues. As the sample is heated further, the lines sharpen, and the overall dispersion gradually decreases, indicating a gradual loss of such structure. Even at 82 °C, however, noticeable deviations from random-coil shifts remain.

Heating of Ca^{2+}-bound equine lysozyme results in no significant effect on the NMR spectrum up to 48 °C. A cooperative unfolding transition is then observed with a midpoint at 55 °C. The chemical shift dispersion at 64 °C, where the first transition is complete, approximates that of the apo protein under similar conditions. Likewise, further heating sharpens the lines and reduces the dispersion, although numerous ring current effects persist to higher temperatures. At 71 °C, both apo and Ca^{2+}-bound proteins display a very similar pattern of aromatic peaks, particularly of upfield-shifted tyrosine resonances, suggestive of an overall similarity of structure under these conditions (Figure 11).

(B) GdnHCl-Induced Unfolding at pH 7.5. Apo equine lysozyme was titrated with GdnHCl in D_2O at pH 7.5, 25 °C (Figures 12 and 13). The introduction of as little as 1 M denaturant to the apo form results in pronounced changes throughout the NMR spectrum (Figure 13). Signals broaden

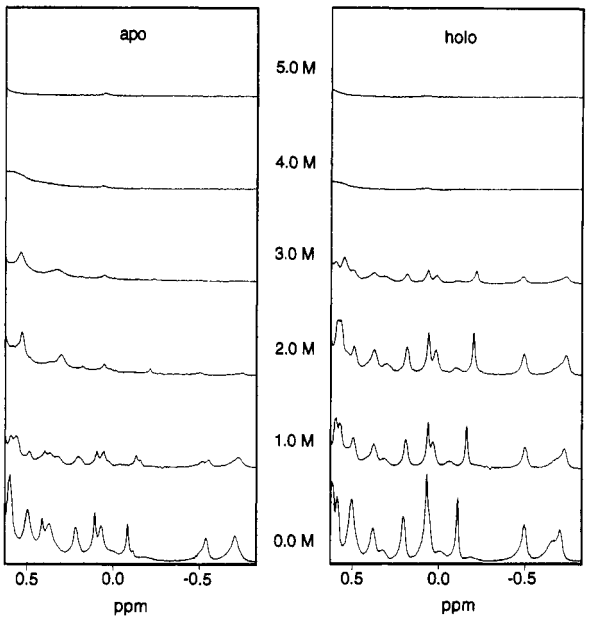

FIGURE 13: NMR spectra of [GdnHCl] dependence of apo (left) and holo (right) equine lysozymes at pH 7.5, 25 °C, from 5.0 M GdnHCl (top) to 0.0 M GdnHCl (bottom).

markedly, and resonances corresponding to the native state diminish in a concerted fashion. In the presence of 3.0 M GdnHCl, the native state resonances are completely absent. Significant chemical shift dispersion remains, however, to the extent that a well-resolved methyl resonance appears as far upfield as 0.33 ppm. Likewise, a large aromatic resonance is found as far upfield as 6.6 ppm (Figure 12). Lines in the aromatic region in particular can be seen to be highly broadened when compared with the spectrum of the apo enzyme at 50 °C, pH 4.5 (Figure 10). Further addition of denaturant results in a sharpening of peaks and a collapse to a more random-coil-like spectrum.

Titration of equine lysozyme with GdnHCl in the presence of 1 mM excess $CaCl_2$ results in little change to the spectrum up to 3.0 M denaturant, at which concentration the native-state resonances become sharply attenuated (Figure 13). This transition appears almost complete in 4.0 M GdnHCl. The spectrum of equine lysozyme under these conditions resembles the corresponding spectrum of the apo protein, in that it retains significant chemical shift dispersion and exhibits extremely broadened lines. As with the apo protein, further addition of GdnHCl results in a decrease in the overall chemical shift dispersion toward frequencies characteristic of random coil.

Differential Scanning Calorimetry. The calorimetric scan of apo equine lysozyme shows two distinct peaks with maxima at 40 and 69 °C (Figure 14). Both these transition zones correspond well with those observed by difference absorption spectroscopy (Figure 7), by NMR (preceding section) and by CD (Morozova et al., 1991). The presence of two peaks unambiguously proves that apo equine lysozyme does not unfold in the highly cooperative manner found for human (Barel et al., 1972) and hen egg white lysozymes (Khechinashvili et al., 1973). It is also clear that apo equine lysozyme is much less stable than human and hen egg white lysozymes which have transition temperatures of 78 and 76 °C, respectively.

The thermal transition is strongly influenced by Ca^{2+} binding. In Figure 15, samples in the Ca^{2+}-bound form (2 and 10 mM Ca^{2+}) show a thermogram with a single asymmetric peak. The transition zone in the Ca^{2+}-bound

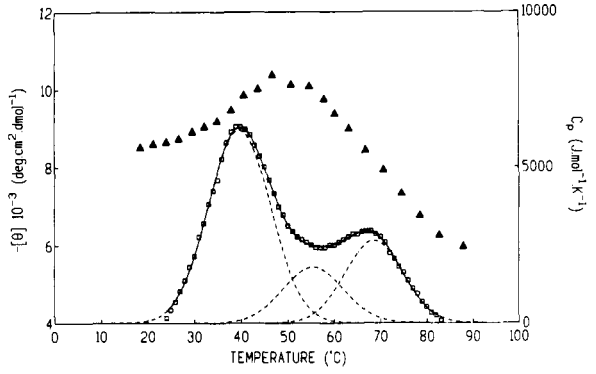

FIGURE 14: Differential scanning calorimetry scan of apo equine lysozyme (right scale): Experimental data (□) are fitted with the sum (full line) of three Gaussian functions (dashed lines). In order to show the relationship with the thermal transitions observed by CD, the ellipticity at 222 nm for apo equine lysozyme, taken from our earlier work (Morozova et al., 1991), is superimposed (▲, left scale).

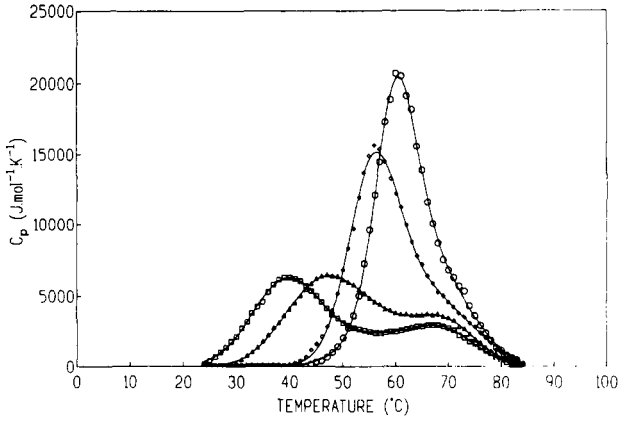

FIGURE 15: DSC scans of equine lysozyme with different Ca^{2+} concentrations: 0 (□), 0.1 (△), 2 (◇), and 10 mM (○). Solid lines represent the fit with three (0 and 0.1 mM Ca^{2+}) or two (2 and 10 mM Ca^{2+}) Gaussian functions. Thermal parameters obtained out of these fits are presented in Table I.

protein is narrow in comparison with the apo form, but still rather broad when compared with those of hen and human lysozymes, suggesting a lesser degree of cooperativity. Figure 15 also shows a thermogram of equine lysozyme in the presence of 0.1 mM Ca^{2+}. Since the logarithm of the binding constant of Ca^{2+} to equine lysozyme is 6.3 or 6.5 as determined by competition experiments with Fura-2 (Nitta et al., 1988) or by batch microcalorimetry (Desmet et al., 1989), respectively, it is clear that in this case the Ca^{2+}-binding site is only partially occupied. In this sample, the first transition is shifted to higher temperature relative to the apo form. The second transition, on the other hand, coincides with the one observed in the absence of Ca^{2+}.

An analysis of the DSC traces by Gaussian deconvolution (Table I) reveals some interesting features of the thermal transition. Despite the appearance of a double maximum in the scan of the apo form, it was impossible to fit the results in a satisfactory way by a combination of only two Gaussian functions. On the other hand, the addition of a third component leads to an excellent fit of the data (Figure 14). It is remarkable that our earlier CD measurements (Morozova et al., 1991), which are also depicted on Figure 14, show a comparable temperature dependence. Although these CD data were originally interpreted as being the result of two instead of three distinct processes, the curve is consistent with the presence of a minor third component with a maximum around 55 °C (Figure 14). An analogous three-component behavior is observed for the sample with low Ca^{2+} content (Figure 15). In the case of the Ca^{2+}-bound protein (Figure 15), however, the first transition disappears, and the DSC curves can be described accurately by a sum of only two Gaussian functions. Peak heights and temperatures at which the maxima occur are represented in Table I, together with the enthalpy changes associated with the various transition phenomena. In the apo form, the first transition has the larger ΔH value; the large value is an indication of the considerable conformational change associated with this step. With increasing Ca^{2+} concentration, the peak height as well as the ΔH value of the first transition decreases in favor of the second transition. For all samples, the latter transition is broad and can be subdivided into two parts. The first peak associated with the second transition is small in the apo form but increases significantly in the Ca^{2+} form, while its maximum shifts from 55 °C up to 60 °C. As the second maximum shifts to lower temperature (69 °C → 64 °C) upon Ca^{2+} addition, we may conclude that Ca^{2+} binding enhances the cooperativity of the unfolding process. The total enthalpy change rises from 174.6 kJ mol^{-1} in the apo state up to 282.4 kJ mol^{-1} in the Ca^{2+}-bound state. It is noteworthy that even in the unstable apo form some residual structure exists that is not lost until above 69 °C.

DISCUSSION

In contrast to other *c*-type lysozymes, equine lysozyme clearly unfolds via at least a two-stage pathway with a well-defined intermediate in the apo state. At pH 4.5, the first thermal transition in the apo enzyme occurs with a midpoint near 40 °C and the second near 70 °C; at pH 7.5, the apo enzyme undergoes a first GdnHCl-induced transition with a midpoint at 0.8 M denaturant and a second one with a midpoint near 3.7 M denaturant. In Ca^{2+}-bound equine lysozyme, the transitions overlap, although the second takes place under conditions similar to the corresponding change in the apo enzyme. Even in the Ca^{2+}-bound enzyme, the overall unfolding transition is quite broad. Thus, equine lysozyme is not only less stable overall than hen or human lysozymes ($T_m \sim 77$ °C) but also unfolds less cooperatively as well.

The parameters for these transitions are closely reproduced by a number of spectroscopic probes that supply complementary information about the nature of the conformational changes at each stage of the unfolding. The CD, fluorescence, and difference absorption results indicate the persistance of secondary structure through the first transition, along with specific changes to the tertiary structure involving the sequestration of aromatic side chains from solvent. NMR spectroscopy shows the first transition to involve two populations in slow exchange, with a loss of intensity in well-dispersed resonances of the native state of the protein coinciding with an intensity gain at frequencies much closer to those expected for an unfolded polypeptide. These results indicate a global loss of highly ordered tertiary structure during the first transition, since the protons giving rise to the resolvable native-state resonances are well distributed throughout the molecule, including both the helical and sheet domains. Nonetheless, the spectrum of the intermediate corresponds poorly to that expected for a random-coil state, inasmuch as it displays substantial chemical shift dispersion and broadened lines. These features are closely reproduced in the spectra of both Ca^{2+}-bound and apo proteins under similar conditions, indicating that Ca^{2+} does not have a significant effect on the conformation of the intermediate state. Under stronger denaturing conditions, the spectrum gradually approaches that

Table I: Thermal Characteristics Derived from the Individual Gaussian Components of the DSC Curves of Equine Lysozyme with Different Ca^{2+} Concentration[a]

Ca^{2+} content (mM)	first transition			second transition						H_{total} (kJ mol^{-1})
	peak height (J K^{-1} mol^{-1})	T_t (°C)	ΔH (kJ mol^{-1})	peak height (J K^{-1} mol^{-1})	T_t (°C)	ΔH (kJ mol^{-1})	peak height (J K^{-1} mol^{-1})	T_t (°C)	ΔH (kJ mol^{-1})	
0	6196	39.7	103.1	1086	55.5	29.7	2646	68.6	41.8	174.6
0.1	5144	45.2	87.4	2889	55.5	52.6	3042	69.3	46.3	186.3
2				11648	59.0	134.0	5485	64.5	148.4	282.4
10				13164	60.0	121.8	8370	64.5	167.8	289.6

[a] Protein concentration = 2.2 mg/mL and pH = 7.5.

characteristic of a random-coil protein. Finally, the noncooperative nature of the unfolding is revealed explicitly by DSC. The first transition in the apo enzyme involves a significantly greater enthalpy change than the second, again in accordance with a global loss of tertiary structure during this transition. Gaussian deconvolution of the DSC curves suggests the presence of a second, minor intermediate that is most evident in the unfolding of the apo protein. The corresponding transitions are not obvious by NMR, however. Gradual changes in line widths and chemical shifts are seen by NMR throughout the range in which the second intermediate is populated, indicating that this species must be in rapid equilibrium with other, more fully unfolded forms.

The extensive spectroscopic results permit a rather detailed description of the nature of the major intermediate state. (For the present, we assume the intermediates generated by GdnHCl or heating to be similar, in light of the numerous parallels in the data for both experiments.) NMR clearly shows the intermediate to be extensively disordered, lacking the persistent and specific side-chain interactions characteristic of a protein in its native state. However, helical secondary structure appears to be largely preserved. Although various conformational substates exchange at a rate too fast for resolvable NMR signals, the markedly broadened resonances observed suggest the exchange to be slower than that seen in a fully unfolded protein, and that a limited number of conformers might be highly populated. The significant degree of residual shift dispersion and the limited degree of changes in the near-UV CD and difference absorption spectra also point to the persistence of some long-range interactions.

The optical data suggest that several tryptophan side chains are buried in the intermediate state, a phenomenon that may be understood as follows. In the native state, some hydrophobic residues may, due to the restricted conformational properties of that state, be exposed to solvent. The partially unfolded intermediate state possesses much greater conformational mobility than the native state, and it is possible for hydrophobic residues to generate a local environment that is effectively removed from bulk solvent. Clustering of hydrophobic residues away from solvent is indeed an energetically favorable process (Dill, 1985). This line of reasoning was recently followed in the analysis of photochemically induced dynamic nuclear polarization (photo-CIDNP) studies of denatured hen lysozyme (Broadhurst et al., 1991). This study revealed nonrandom hydrophobic interactions, particularly involving tryptophan residues, that gave rise to hydrophobic clusters within the denatured state. Because tryptophan is more hydrophobic than tyrosine, the former residue is more likely to participate in the clustering process.

The picture of the equine lysozyme unfolding intermediate presented by the data corresponds in many respects to that of a classical molten globule. Studies of α-lactalbumins in particular have defined the molten globule as a compact denatured state with extensive secondary structure. The global nature of the initial denaturation in equine lysozyme is in accordance with such a state, as opposed to the regionally denatured intermediates observed in the unfolding of some large multidomain or modular proteins (Oswald et al., 1989). Despite the evidence that hen lysozyme at least has two distinct folding domains (Miranker et al., 1991; Radford et al., 1992), it is clear that the first unfolding transition of equine lysozyme does not correspond to the loss of structure in one of these domains while the fully nativelike structure is maintained in the other. Like α-lactalbumins, the unfolding of equine lysozyme depends critically on the metal-binding state of the enzyme. Binding of Ca^{2+} enhances the cooperativity of unfolding in both proteins, presumably by stabilizing the native state (Desmet et al., 1989). There is no evidence, however, from any detectable effects on its stability or spectral properties that Ca^{2+} binds to the equine lysozyme intermediate, in contrast to the situation for an intermediate formed in the refolding of subtilisin lacking its pro-sequence (Eder et al., 1993).

In at least two important respects, however, the equine lysozyme intermediate differs from the α-lactalbumin molten globule. To begin with, the near-UV CD changes in the first transition of equine lysozyme are much less pronounced than those observed in α-lactalbumins. The major transition in the near-UV is in fact the second one, which coincides with the loss of secondary structure monitored in the far-UV. In α-lactalbumins, the main transition in the near-UV clearly precedes that in the far-UV (Ikeguchi et al., 1986; Kuwajima, 1989). Secondly, the DSC results show that the second unfolding transition in apo equine lysozyme involves a significant enthalpy change of more than two-thirds the magnitude of the first one. The α-lactalbumin molten globule, in contrast, shows little excess heat absorption during unfolding to the U-state (Kuwajima, 1989; Yutani et al., 1992). This observation must be interpreted cautiously, inasmuch as the thermal transition of molten globules may be broadened and reduced below the point of detection under certain conditions (Haynie & Freire, 1993). Nonetheless, taken together, our results are consistent with an intermediate state of equine lysozyme that is more nativelike than the α-lactalbumin molten globule. The minor transition identified by DSC and CD might then be interpreted to reflect the hierarchical nature of residual structure in the equine lysozyme intermediate.

The identification of a stable partly unfolded state of equine lysozyme has important ramifications for our understanding of the folding mechanism in this family of enzymes. Examination of the folding of hen lysozyme has shown that the protein is not organized in a single cooperative event but that different parts of the structure become stabilized with very different kinetics (Radford et al., 1992). The availability of a stable, well-resolved intermediate in the unfolding of equine lysozyme will permit detailed exploration of the nature and location of structure in a partly unfolded state of a closely related protein and may shed considerable light on the nature

of the distinct folding domains detected during the kinetic refolding of hen lysozyme (Radford et al., 1992). Preliminary hydrogen exchange results, for example, have shown significant protection of amide NH protons in the helical domain of native and acid-denatured equine lysozyme (Arico-Muendel et al., unpublished results). Since formation of the intermediate may be controlled by varying the Ca^{2+} concentration, a number of trapping experiments, in addition to those involving pH jump (Baum et al., 1989), are possible, and they will allow the study of amide exchange properties of the thermally- and GdnHCl-induced intermediates in their native states; such approaches have recently been demonstrated with apomyoglobin (Hughson et al., 1990). The results of such studies are likely to be of general significance in understanding the mechanism of protein folding.

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