Protein Dissection Experiments Reveal Key Differences in the Equilibrium Folding of α-Lactalbumin and the Calcium Binding Lysozymes†

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ABSTRACT: The α-lactalbumins and c-type lysozymes have virtually identical structure but exhibit very different folding behavior. All α-lactalbumins form a well populated molten globule state, while most of the lysozymes do not. α-Lactalbumin consists of two subdomains, and the α-subdomain is considerably more structured in the molten globule state than the *β*-subdomain. Constructs derived from the α-subdomain of human α-lactalbumin containing the A, B, D, and 3_{10} helices are known to form a molten globule state in the absence of the rest of the protein (Demarest, S. et al. (1999) *J. Mol. Biol. 294,* 213−221). Here we reported comparative studies of constructs derived from the same regions of canine and equine lysozymes. These proteins form two of the most stable molten globule states among all the lysozymes. A construct containing the A, B, D, and 3_{10} helices of equine lysozyme is partially helical but is less structured than the corresponding human α-lactalbumin peptide. Addition of the C-helix leads to a construct that is still less structured and less stable than the α-lactalbumin construct. The corresponding construct from canine lysozyme is also less structured and less stable than the α-lactalbumin peptide. Thus, molten globule formation in human α-lactalbumin can be driven by the isolated α-subdomain, while more extensive interactions are required to generate a stable molten globule in the two lysozymes. The stability of the canine and equine lysozyme constructs is similar, indicating that the extraordinary stability of the canine lysozyme molten globule is not due to an unusually stable isolated α-subdomain.

A significant number of proteins form partially folded states that are stable under mild denaturing conditions (*1*−*7*). These states are typically known as molten globules and in many cases are thought to mimic partially folded intermediate states populated along the folding pathway (*1*−*5*, *8*−*10*). Consequently, there has been considerable interest in elucidating the interactions that stabilize the molten globule and lead to its formation. Interest in partially folded states has been heightened by the realization that some proteins aggregate to form amyloid from partially folded precursors and by the recognition that many designed proteins populate states that share some of the properties of the molten globule (*11*). The classic definition of the molten globule state is a state that is almost as compact as the native state and possesses a significant amount of presumably nativelike secondary structure but lacks most fixed tertiary interactions characteristic of fully folded proteins (*3*, *6*, 7). A variety of molten globule states have been characterized in a range of proteins. Some are more structured than the originally defined molten globule and contain tertiary interactions. Others, in contrast, have been shown to be less structured. An additional partially folded state, the premolten globule, has been proposed, which has properties between the molten globule and unfolded state (*12*, *13*). The determination of the structures and the interactions, which stabilize partially folded states, is an active area of research.

This paper is concerned with the origin of the difference in the equilibrium unfolding behavior of human α-lactalbumin and calcium binding variants of the c-type lysozymes. The α-lactalbumins and c-type lysozymes have essentially identical folds and very high sequence identity and are believed to be evolutionary related (*14*−*16*). α-Lactalbumin is a calcium-binding protein, as are some but not all of the c-type lysozymes. The calcium binding lysozymes are thought to be an evolutionary link between the α-lactalbumins and non-calcium binding c-type lysozymes. The lysozymes and α-lactalbumins display very different folding behavior despite their sequence similarity and identical folds (*1*−*3*, *14*, *17*, *18*). All of the α-lactalbumins studied to date form a stable equilibrium molten globule state. The human protein forms one of the most stable molten globule states among members of this class of proteins. In contrast, many of the c-type lysozymes do not form equilibrium molten globule states (*14*). Notable exceptions include the calcium binding c-type lysozymes, equine lysozyme, and canine lysozyme (*19*−*23*). The equine and canine variants of

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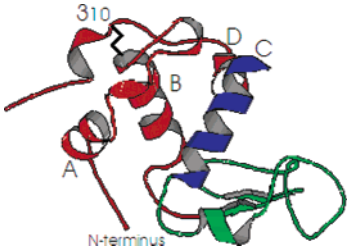

FIGURE 1: Ribbon diagram of the lysozyme structure. The region corresponding to the AB-D/3_{10} construct is shown in red. The C-helix is in blue, and the AB-CD/3_{10} construct is shown in red and blue. Only the 30−115 disulfide bond is shown. The major helices and N-terminus are labeled. The diagram was constructed using the program MOLSCRIPT (*41*) and the PDB file for equine lysozyme.

lysozyme appear to form the most stable molten globule states among members of the lysozyme family, with the canine lysozyme molten globule being significantly more stable than the equine lysozyme molten globule (*18*, *20*, *21*, *23*). Experiments with chimeras have shown that the ability to form a stable molten globule is not simply due to the presence of a calcium binding site (*24*). The reason for the differences in the folding behavior of different members of the lysozyme/α-lactalbumin family is still not clear.

The lysozyme/α-lactalbumin fold consists of two subdomains (Figure 1). The *β*-subdomain contains a small *β*-sheet and several loops. The α-subdomain is rich in helical structure and includes the A, B, D, and C-terminal 3_{10} helices. The relatively long C-helix lies at the interface of the subdomains and is assigned by some workers to the α-subdomain and by others to the *β*-subdomain (*25*). There is a conserved pattern of disulfides in these proteins. The 6−127 disulfide (lysozyme numbering) links the N and C-terminal regions but is not essential for maintaining the fold (*26*). The 30−115 disulfide (lysozyme numbering) is located in the α-subdomain and links the B-helix to the D/3_{10} helical region. One disulfide, the 64−80, resides entirely in the *β*-subdomain, while the fourth connects Cys-76 to Cys-94 and links the C-helix to the *β*-subdomain. The calcium-binding site is formed by an "elbow" involving residues at the N-terminus of the C-helix and in the loop before the start of this helix.

The molten globule state of α-lactalbumin has been studied in detail. Under acidic conditions, the 123 residue protein adopts a molten globule state characterized by a disordered or partially ordered *β*-subdomain, whereas the α-subdomain retains substantial helical secondary structure (*1*, *2*, *27*−*31*). Studies performed by Kim and co-workers with human α-lactalbumin have shown that a covalent model of the isolated α-subdomain (residues 1−39 and 81−123 connected by a glycine linker) folds into a molten globule-like structure in the absence of the *β*-subdomain (*27*). Work from our laboratory has shown that the C-helix is not required and has demonstrated that a construct consisting of the AB helix region cross-linked by the native 28 to 111 disulfide (α-lactalbumin numbering) to the D/3_{10} helix region forms a stable molten globule state, which is rich in helical structure (*32*−*35*). These studies have defined the minimum structured core of the human α-lactalbumin molten globule. A similar peptide construct from hen lysozyme was shown to be largely unstructured, which is expected, since full-length hen lysozyme does not form a molten globule state (*32*).

In this work, we examine the folding behavior of α-subdomain constructs derived from canine and equine lysozyme and compare them to our previous studies of the related constructs from human α-lactalbumin (Figure 2) (*32*, *35*). A 40-residue-long peptide that includes the A and B helices of equine lysozyme was cross-linked via the 30−115 disulfide (lysozyme numbering) to a 30 residue peptide containing the D and 3_{10} helices of equine lysozyme. The construct contains all of the residues previously identified as comprising the minimum core unit of the human α-lactalbumin molten globule but lacks the C-helix. A larger construct that includes the C-helix was characterized to examine the role of this helix, as was the related construct from canine lysozyme. The canine lysozyme construct includes the residues recently proposed to play a critical role in stabilizing the canine lysozyme molten globule (*21*, *36*). Comparison of the properties of these molecules with the previously studied constructs from human α-lactalbumin provides new insight into the factors that stabilize molten globule states in the calcium binding lysozymes.

MATERIALS AND METHODS

Peptide Synthesis and Purification. Peptide synthesis was performed using a 9050 Millipore peptide synthesizer. Amino acid derivatives were purchased from PerSeptive Biosystems Inc. and Advanced ChemTech, and the solvents were

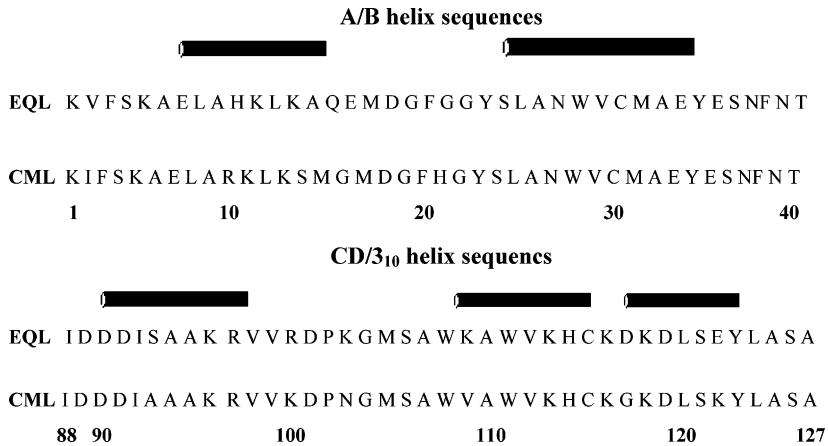

FIGURE 2: Primary structures of the helical subdomains of Equine (EQL) and Canine (CML) lysozyme. Cylinders above the sequences indicate the location of the native helices in the primary sequence. The standard alignment of Acharya and co-workers was used (*42*). Top, the AB helix region. Bottom, the CD/3_{10} helix region.

purchased from Fisher Scientific. Peptides were prepared on a 0.200-mmol scale using standard Fmoc cycles for peptide synthesis. In the AB and CD/3_{10} peptides, the wild-type Cys residues at positions 6 and 127 were substituted with Ala. Activation steps were carried out using HATU and HBTU as coupling reagents. PAL-PEG resin was used, which provides a C-terminal amide. The amino termini of the AB peptides were not acetylated, but the amino termini of the other peptides were. Cleavage from the resin was accomplished using a cleavage cocktail containing 90% TFA, 3.3% ethanedithiol, 3.3% anisole, and 3.3% thioanisole. Peptides were purified by reverse phase HPLC with a Vydac C18 column. The composition of the buffers used for HPLC were A = 100% H_2O and 0.045% HCl and B = 20% H_2O and 80% CH_3CN with 0.045% HCl. The correct synthesis of each peptide was confirmed by MALDI TOF mass spectrometry. The peptide fragments were cross-linked via disulfide formation induced by air oxidation for 24 h at pH 8.5 and purified by reversed-phase HPLC. All of the oxidation reactions led to a simple pattern of five peaks on the HPLC trace that represents two homodimers, the desired heterodimer, and the two unreacted monomers. The peaks were identified by MALDI TOF mass spectrometry. The peptide constructs and reduced peptide fragments were all greater than 95% pure, as judged by analytical HPLC.

Circular Dichroism. An Aviv Model 62A circular dichroism spectrometer was used to perform CD[1] measurements. An averaging time of 3 s was used for wavelength scans, which were taken with a minimum of five repeats. For far UV CD scans, a cuvette of 1-mm path length and 250-μL sample volume was used. A standard buffer of 2 mM phosphate (sodium phosphate, monobasic), 2 mM citrate (citric acid, anhydrous) and 2 mM borate (sodium borate) with 10 mM NaCl was used. Concentration-dependent experiments were performed by dilution from a concentrated stock solution into the buffer described above. The concentration of the stock solutions was determined by measuring the absorbance of the peptide at 280 nm, using extinction coefficients calculated by use of the program Expassy protoparam. All CD and fluorescence experiments were performed at pH 2.8, 25 °C, unless otherwise stated in the text, to allow direct comparison to previous studies.

Fluorescence Measurements. An ISA Fluorolog spectrometer was used to perform fluorescence measurements. Trp fluorescence emission measurements were performed using an excitation wavelength of 280 nm. The spectra were recorded over the range of 290−500 nm. The ANS spectra were recorded over the range of 380−650 nm. The excitation wavelength for ANS fluorescence experiments was 370 nm. The ANS concentration was measured by using a molar absorption coefficient of 7.8×10^3 M^{-1} cm^{-1} at 372 nm in methanol (provided by Molecular Probes). The stock solution was diluted into buffer at pH 2.8, yielding a final ANS concentration of 2 μM at 25 °C. Similar experimental conditions were used at pH 7.0.

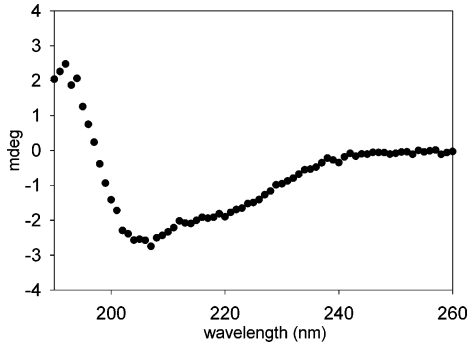

FIGURE 3: CD spectra of the oxidized equine lysozyme construct, EQL AB-D/3_{10}, recorded at pH 2.8 and 25 °C.

Sedimentation Equilibrium. Sedimentation equilibrium experiments were performed at 25 °C using a Beckman XL-A analytical ultracentrifuge (Haverford College) or XL-I analytical ultracentrifuge (Stony Brook University). Experiments on the EQL AB-D/3_{10} construct were performed at two concentrations, 20 and 50 μM. The EQL AB-CD/3_{10} construct was studied at 5, 10, and 25 μM, while the CML AB-CD/3_{10} construct was studied at 3, 14, and 38 μM. All experiments were conducted at 25 °C. These experiments were carried out using 12-mm path length, six-channel, charcoal-filled, Epon cells with quartz windows. Partial specific volumes were calculated from the weighted average of the partial specific volumes of the individual amino acids. The data were fit with a single species model with the molecular weight treated as a fitting parameter.

RESULTS

A Construct Containing the A, B, D, and 3_{10} Helices of Equine Lysozyme is Partially Structured. Our previous studies have demonstrated that the minimum core of the human α-lactalbumin molten globule is made up of the A, B, D, and 3_{10} helices (*35*). A similar construct derived from equine lysozyme was prepared, to determine if the same region constitutes the minimum core required to generate a stable molten globule state in equine lysozyme. The A and B helices were synthesized as one unit consisting of residues 1−40 of equine lysozyme. The peptide is designated EQL AB. A 30-residue peptide denoted EQL D/3_{10} was prepared that contains the D and C-terminal 3_{10} helices. This peptide corresponds to residues 98−127 of equine lysozyme. Cross-linking the two peptides via the native 30−115 disulfide bond did not lead to any noticeable increase in structure, as judged by CD. In contrast, cross-linking the two human α-lactalbumin peptides do lead to a significant increase in structure (*35*). The CD spectrum of EQL AB-D/3_{10} indicates partial helical structure (Figure 3). The spectrum is independent of

[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; CD, circular dichroism; CML AB-CD/3_{10}, a peptide consisting of residues 1−40 of canine milk lysozyme cross-linked via the 30−115 disulfide to a peptide consisting of residues 88−127 of canine lysozyme; EQL AB, a peptide corresponding to residues 1−40 of equine lysozyme; EQL D/3_{10}, a peptide corresponding to residues 98−127 of equine lysozyme; EQL CD/3_{10}, a peptide corresponding to residues 88−127 of equine lysozyme; EQL AB-D/3_{10}, a peptide corresponding to residues 1−40 of equine lysozyme cross-linked via the 30−115 disulfide to a peptide consisting of residues 98−127 of equine lysozyme; EQL AB-CD/3_{10}, a peptide corresponding to residues 1−40 of equine lysozyme cross-linked via the 30−115 disulfide to a peptide consisting of residues 88−127 of equine lysozyme; GnHCl, guanidinium hydrochloride; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-b]pyridine-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HPLC, high-pressure liquid chromatography; MALDI TOF, matrix assisted laser desorption ionization time-of-flight mass spectrometry; PAL PEG, 5-(4′-Fmoc-aminomethyl-3′, 5′−dimethoxyphenoxy) valeryl poly(ethylene glycol); TFA, trifluoacetic acid.

concentration over the range studied, 5−100 μM. Analytical ultracentrifugation experiments conducted at 20 and 50 μM peptide concentrations confirm that the construct is monomeric. The EQL AB-D/3_{10} construct is partially helical under acidic conditions, but is less structured than the AB-D/3_{10} construct of human α-lactalbumin, although it is more structured than a similar peptide derived from hen lysozyme (*32*). Molten globule states can often be formed under a range of conditions, and their stability can vary; consequently, we also examined the construct at pH 7.0. No significant change in the shape of the CD spectrum is observed at pH 7.0. The AB-D/3_{10} construct was also analyzed in the presence of Na_2SO_4, a well-known stabilizing agent. The addition of 400 mM Na_2SO_4 leads to a small, 15−20% increase in the value of θ_{222}; however, the construct is still less structured in the presence of sulfate than the human α-lactalbumin construct is in the absence of any stabilizing agent.

Fluorescence emission experiments are consistent with the CD studies. EQL AB-D/3_{10} contains all three of the Tyr and three of the five Trp residues of equine lysozyme. The emission maximum of EQL AB-D/3_{10} is 349 nm, which is red shifted from the emission maximum in the intact molten globule. The emission maximum shifts by only 3 nm to 352 nm in the presence of the denaturant GnHCl. This result indicates that the Tyr and Trp residues in EQL AB-D/3_{10} are not significantly sequestered from solvent. ANS dye binding studies were also performed to probe the properties of the equine lysozyme peptide. ANS is a hydrophobic dye that contains a sulfonate group and binds to molten globule states (*37*). ANS binding is often used as a test for the formation of a loosely packed hydrophobic core, although electrostatic interactions can contribute to the binding (*38*). A solution containing 2 μM ANS showed almost a 10-fold increase in the fluorescence intensity in the presence of 4 μM EQL AB-D/3_{10}. The binding observed for the equine peptide construct could be due to the transient hydrophobic clusters or it may result from electrostatic interactions between the negatively charged sulfonate on ANS and the positively charged peptide. At low pH, the estimated net charge is about 12 for EQL AB-D/3_{10}. We also note that the observation of ANS binding does not require formation of a molten globule state. For example, constructs derived from hen lysozyme have been shown not to form a molten globule, yet they show a similar enhancement in ANS fluorescence (*32*).

An Equine Lysozyme Construct Containing the A, B, D, 3_{10}, and C-Helices is Still Less Structured than the Human α-Lactalbumin AB-D/3_{10} Construct. The EQL AB-D/3_{10} construct lacks the C-helix. This helix lies at the interface of the α and β subdomains and packs against the B and D helices. Thus, it is possible that our initial equine lysozyme construct was partially structured because it lacked the C-helix. A larger peptide was prepared, to test this possibility and to examine the role of the C-helix in stabilizing the isolated α-subdomain. This 80 residue construct is made up of the AB peptide, residues 1−40, cross-linked by the native 30−115 disulfide to a peptide comprised of residues 88−127. The complete construct is designated EQL AB-CD/3_{10}, and the C-terminal segment is denoted as EQL CD/3_{10}. EQL CD/3_{10} is only modestly structured as judged by CD. EQL AB has a tendency to associate, but at low concentration, the mean residue ellipticity is −7000 deg cm^2 dmol^{-1} at 222 nm, pH 2.8, and 25 °C. The value of θ_{222} for the isolated D/3_{10} peptide is −5000 deg cm^2 dmol^{-1}. Cross-linking the AB peptide to EQL CD/3_{10} leads to a partially helical heterodimer (Figure 4A). Comparison of the far UV CD spectrum of EQL AB-CD/3_{10} to the spectrum calculated from the normalized sum of the spectra of the reduced peptides indicates that the helicity is greater than that expected from the sum of the reduced peptides. The estimated mean residue ellipticity of the oxidized dimer is −11000 deg cm^2 dmol^{-1}, compared to the value of −6000 deg cm^2 dmol^{-1} calculated from the normalized sum of the reduced peptides CD signal. Analytical ultracentrifugation experiments conducted at 5, 10, and 25 μM peptide concentrations indicate that the oxidized heterodimer, EQL AB-CD/3_{10} is monomeric. In addition, the CD spectra of the oxidized peptide construct were independent of concentration over the concentration range studied at pH 2.8, 5−100 μM. By use of the method of Rohl and Baldwin, the apparent fraction helix was calculated to be 28% (*39*). This value could be generated if 21 of the 80 residues were fully helical and the rest were random. Although this is physically unreasonable, it provides a way to compare with previous studies. For example, in the case of the human α-lactalbumin construct, the calculated fraction helix is 34%. This value could be generated if 22 of the 64 residues in that peptide were fully helical. Published CD spectra of the low pH form of the intact equine lysozyme molten globule give values of θ_{222} between −10 000 and −12 000, corresponding to 27−31% apparent fraction helix

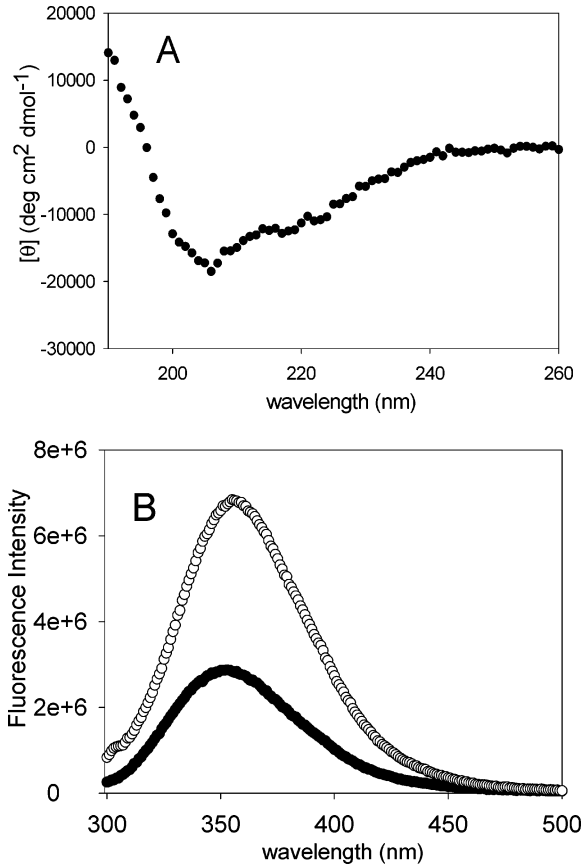

FIGURE 4: CD and fluorescence emission spectra of the oxidized equine lysozyme construct EQL AB-CD/3_{10}. (A) CD spectrum recorded at 4 μM, pH 2.8 and 25 °C. (B) Fluorescence emission spectra. Closed circles: data collected at pH 2.8 and 25 °C, no denaturant. Open circles: data collected in the presence of 6 M GnHCl, pH 2.8, and 25 °C.

(*19*, *20*, *29*). This level of helical structure would be generated if 35−40 of the residues were helical and the rest were random. This analysis is clearly semiquantitative at best, but it illustrates two important points: first, that the equine lysozyme α-subdomain construct is less structured than that of human α-lactalbumin construct, and second, the helicity of the equine lysozyme construct cannot account for the known amount of helical structure in the molten globule formed by the intact protein.

The CD spectrum recorded at pH 7.0 is similar to that observed at acidic pH, indicating that there is little or no change in helical content. The EQL AB-CD/3_{10} construct was also studied in the presence of Na_2SO_4. A small enhancement of 10% in the mean residue ellipticity is observed in the presence of 400 mM Na_2SO_4. The helicity of this construct, while higher than that observed for EQL AB-D/3_{10}, is still less than that of the human α-lactalbumin construct in the absence of sulfate; even though the human α-lactalbumin peptide lacks the C-helix.

The fluorescence emission maximum of EQL AB-CD/3_{10} is 353 nm at acidic pH. Addition of 6 M GnHCl shifts the emission maximum to 355 nm (Figure 4B). The results are similar to those observed for the construct, which lacks the C-helix and indicates that the Trp and Tyr residues are exposed. The construct binds ANS, and the enhancement of ANS fluorescence is comparable to that observed with the shorter construct.

Analysis of the α-Subdomain of Canine Lysozyme Shows that it Does Not Form a Well-Structured State in Isolation. The experiments described above show that isolated subdomain constructs derived from equine lysozyme are less structured and less stable than those from human α-lactalbumin. We examined an α-subdomain construct from another calcium binding lysozyme, to test if the results were peculiar to equine lysozyme or are more general. Canine lysozyme was chosen because the intact protein forms the most stable molten globule state among the calcium binding lysozymes studied to date (*21*, *36*). Comparison of the equine and canine constructs also allows us to test if the extraordinary stability of the canine molten globule is due to a very stable isolated α-subdomain.

We designed a canine lysozyme peptide model that was similar to our larger equine lysozyme construct. The increased stability of the canine molten globule compared to the equine molten globule has been ascribed to specific packing interactions in the α-subdomain involving His21 in the turn between the A and B helices and a hydrophobic cluster rich in aromatics around Val-98 and Met-105 (*36*). A second difference between the proteins that is thought to be important is in the D-helix, where side chain−side chain interactions are considered to be more favorable in the canine protein (*21*). Our construct contains all of these residues; thus, comparing its stability to that of EQL AB-CD/3_{10} will indicate if the increased stability of the canine lysozyme molten globule is caused by an exceptionally stable α-subdomain. The canine construct is designated CML AB-CD/3_{10} and is made up of two peptides linked by the native 30−115 disulfide. The first fragment contains residues 1−40, and the second contains residues 88−127. Analytical ultracerifugation experiments indicate that the oxidized heterodimer is monomeric at low (3 μM) concentration and has a slight tendency to associate at higher concentrations (14 μM and 38 μM). For example, the apparent molecular weight determined from the best fit single species model at 14 μM is 25−37% larger than the true value. The CD spectrum of the oxidized canine lysozyme construct is similar to the one recorded for the related equine lysozyme construct (Figure 5A). Partial helical structure is observed, and the signal is larger than that calculated from the normalized sum of the reduced peptides. However, like the equine lysozyme constructs, CML AB-CD/3_{10} is less structured than the shorter human α-lactalbumin peptide. CD spectra recorded at pH 7.0 are similar to those measured at low pH. The mean residue ellipticity at 222 nm increases by 25% in the presence of Na_2SO_4 but again is still less than that observed for the shorter human α-lactalbumin construct in the absence of sulfate.

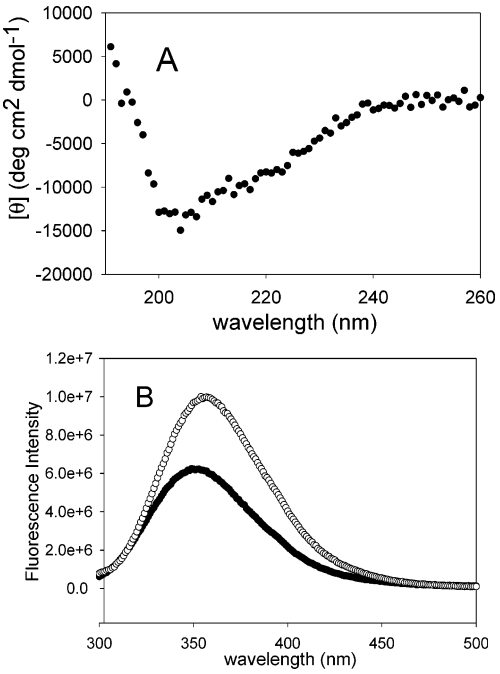

FIGURE 5: CD and fluorescence emission spectra of the oxidized canine lysozyme construct CML AB-CD/3_{10}. (A) CD spectrum recorded at 5 μM, pH 2.8, and 25 °C. (B) Fluorescence emission spectra. Closed circles: data collected at pH 2.8 and 25 °C, no denaturant. Open circles: data collected in the presence of 6 M GnHCl, pH 2.8, and 25 °C.

The Trp emission maximum for CML AB-CD/3_{10} is 349 nm at low pH in the absence of denaturant. This construct contains two of three Tyr residues and three of the five Trp residues of canine lysozyme. In the presence of GnHCl, the emission maxima shifts to 354 nm (Figure 5 B). These results are very similar to those obtained with the equine constructs and indicate that the Trp and Tyr residues are not well protected from solvent. ANS dye binding studies were also performed. A solution containing 2 μM ANS at pH 2.8 showed almost a 4-fold increase in fluorescence intensity in the presence of 4 μM peptide. Similar behavior was observed at pH 7.0. Again, the results are similar to those obtained for the equine lysozyme constructs.

The Equine and Canine Lysozyme Constructs Are Less Stable than the Human α-Lactalbumin Construct. The urea-induced unfolding of the equine and canine lysozyme AB-CD/3_{10} constructs was followed by CD. The signal at 222 nm is largely eliminated at high urea concentrations, and the transition is steep, with 50% of the initial signal lost by

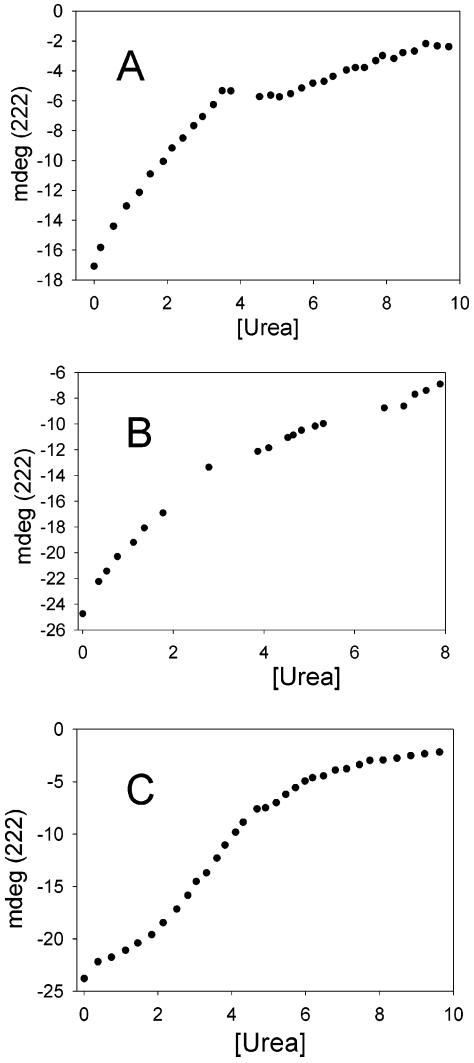

FIGURE 6: Urea-induced unfolding monitored by CD at 222 nm. (A) the oxidized equine lysozyme construct, EQL AB-CD/3_{10}, (B) the oxidized canine lysozyme construct, CML AB-CD/3_{10}, and (C) the oxidized human α-lactalbumin construct AB-D/3_{10}, which consists of residues 1−38 cross linked to residues 95−120. Data for (C) are adapted from Demarest et al. (*33*). Experimental conditions were pH 2.8 and 25 °C.

less than 2 M urea (Figure 6). The shorter human α-lactalbumin construct is much more resistant to urea-induced unfolding and displays a sigmoidal unfolding curve with a pretransition, which is lacking in the larger lysozyme constructs. The apparent midpoint of the unfolding curve for the human α-lactalbumin peptide has been previously determined to be 3.5 M urea (*33*). These results show that the lysozyme constructs are less stable than the human α-lactalbumin construct, as well as less structured. Furthermore, the stability of the two lysozyme constructs appears to be fairly similar as judged by the resistance to urea denaturation.

CONCLUSIONS

The α-subdomains of human α-lactalbumin, equine, and canine lysozymes are known to be structured in the molten globule states formed by the intact proteins. The work presented here, however, shows that there are significant differences in the behavior of the isolated subdomains. The α-subdomain from human α-lactalbumin forms a molten globule in isolation that is somewhat less stable than the molten globule formed by the intact protein but is highly structured and exhibits a sigmodial urea denaturation curve (*33*, *40*). In contrast, the equine and canine lysozyme constructs are less structured and less resistant to urea denaturation, even though they contain the C-helix, while the α-lactalbumin peptide does not. The stability and high helical content of the human α-lactalbumin α-subdomain construct indicates that molten globule formation in this system can be rationalized on the basis of a structured α-subdomain that can fold autonomously. In contrast, molten globule formation in the calcium binding lysozymes is more cooperative in the sense that more of the protein chain is required to generate a stable molten globule. The molten globule states of canine and equine lysozymes are thus stabilized by a mechanism that must differ from that of human α-lactalbumin. Thus, the interactions that stabilize the folding intermediates of the calcium binding lysozyme/α-lactalbumin family are not strictly conserved, suggesting that rigorous conservation of folding intermediates may not be critical in the evolution of these proteins.

The lower helical content and reduced stability of the two lysozyme constructs indicate that interactions involving the *β*-subdomain and possibly the 6−127 disulfide bond are important. This correlates well with earlier amide H/D exchange experiments. Amide H/D exchange studies indicate that the *β*-subdomain is at least partially structured in the molten globule states populated by the canine and equine lysozymes but is much less structured in the human α-lactalbumin molten globule (*19*, *31*, *36*). Furthermore, the *β*-subdomain of the canine lysozyme displays significantly greater protection against exchange than the *β*-subdomain of equine lysozyme in the molten globule.

The urea unfolding experiments show that the isolated α-subdomain constructs of canine and equine lysozymes are approximately equally susceptible to denaturant-induced unfolding. This is an interesting observation because it shows that the much greater stability of the canine molten globule is not due to an intrinsically more stable α-subdomain. Previous studies have highlighted differences in interactions within the α-subdomains of canine and equine lysozymes (*21*, *36*). Our results show that these interactions are not sufficient to lead to a large difference in the stability of the isolated α-subdomains. These observations can be reconciled with the earlier studies because the region in question is near the α−*β* subdomain interface and residues from the *β*-subdomain pack against it. These interactions will stabilize the α-subdomain in the intact protein but are absent in the construct. Taken together, our analysis of the isolated α-subdomains and previous studies of the intact proteins provide a complimentary picture of potential factors that contribute to the significant difference in stability between the canine and equine lysozyme molten globules. Our results highlight the roles played by the *β*-subdomain and by α-subdomain−*β*-subdomain interactions in the folding of the calcium binding lysozymes.

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REFERENCES

1. Kuwajima, K. (1989) The molten globule state as a clue for understanding the folding and cooperatively of globular-protein structure. *Proteins*: *Struct., Funct., Genet. 6*, 87−103.
2. Creighton, T. E. (1997) How important is the molten globule for correct protein folding? *Trends Biochem. Sci. 22*, 6−10.
3. Ptitsyn, O. B. (1995) Molten globule and protein folding. *Adv. Prot. Chem. 47*, 83−217.
4. Arai, M., and Kuwajima, K. (2000) Role of the molten globule state in protein folding. *Adv. Protein Chem. 53*, 209−271.
5. Fink, A. L. (1995) Compact intermediate states in protein folding. *Annu. Rev. Biomol. Struct. 24*, 495−522.
6. Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S. Y., and Ptitsyn, O. B. (1981) α−Lactalbumin: compact state with fluctuating tertiary structure? *FEBS Lett. 136*, 311−315.
7. Ohgushi, M. a. W., A. (1983) "Molten globule state": a compact form of globular proteins with mobile side-chains. *FEBS Lett. 164*, 21−24.
8. Jennings, P. A., and Wright, P. E. (1993) Formation of a molten globule intermediate early in the kinetic folding pathway of apomyoglobin. *Science 262*, 892−896.
9. Chamberlain, A. K., and Marqusee, S. (2000) Comparison of equilibrium and kinetic approaches for determining protein folding mechanisms. *Adv. Prot. Chem. 53*, 283−328.
10. Engelhard, M., and Evans, P. A. (1995) Kinetics of interaction of partially folded proteins with a hydrophobic dye: Evidence that molten globule character is maximal in early folding intermediate. *Protein Sci. 4*, 1553−1562.
11. Kelly, J. W. (1998) The alternative conformations of amyloidogenic proteins and their multistep assembly pathways. *Curr. Opin. Struct. Biol. 8*, 101−106.
12. Uversky, V. N., and Ptitsyn, O. B. (1994) "Partly folded" state, a new equilibrium state of protein molecules: four-state guanidinium chloride-induced unfolding of *β*-lactamase at low temperature. *Biochemistry 33*, 2782−2791.
13. Uversky, V. N., and Ptitsyn, O. B. (1996) Further evidence on the equilibrium "pre-molten globule state": four-state guanidinium chloride-induced unfolding of carbonic anhydrase B at low temperature. *J. Mol. Biol. 255*, 215−228.
14. Qasba, P. K., and Kumar, S. (1997) Molecular divergence of lysozymes and α−lactalbumin. *CRC Crit. Rev. Biochem. 32*, 255−306.
15. Acharya, K. R., Ren, J., Stuart, D. I., Phillips, D. C., and Fenna, R. E. (1991) Crystal structure of human α−lactalbumin at 1.7 Å resolution. *J. Mol. Biol. 221*, 571−581.
16. Grobler, J. A., Ramakrishna, R. K., Pervaiz, S., and Brew, K. (1994) Sequences of two highly different canine type C lysozymes: implications for the evolutionary origins of the lysozyme/α−lactalbumin superfamily. *Arch. Biochem. Biophys. 313*, 360−366.
17. Dobson, C. M., Evans, P. A., and Radford, S. E. (1994) Understanding how proteins fold: the lysozyme story so far. *Trends Biochem. Sci. 19*, 31−37.
18. Ikeguchi, M., Kuwajima, K., Mitani, M., and Sugai, S. (1986) Evidence for identity between the equilibrium unfolding intermediate and a transient folding intermediate: a comparative study of the folding reactions of α-lactalbumin and lysozyme. *Biochemistry 25*, 6965−6972.
19. Morozova-Roche, L. A., Arico-Muendel, C. C., Haynie, D. T., Emelyanenko, V. I., Van Dael, H., and Dobson, C. M. (1997) Structural characterization and comparison of the native and A-state of equine lysozyme. *J. Mol. Biol. 268*, 903−921.
20. Mizuguchi, M., Arai, M., Ke, Y., Nitta, K., and Kuwajima, K. (1998) Equilibrium and kinetics of the folding of equine lysozyme studied by circular dichroism spectroscopy. *J. Mol. Biol. 283*, 265−277.
21. Koshiba, T., Yao, M., Kobashigawa, Y., Demura, M., Nakagawa, A., Tanaka, I., Kuwajima, K., and Nitta, K. (2000) Structure and thermodynamics of the extraordinarily stable molten globule state of canine milk lysozyme. *Biochemistry 39*, 3248−3257.
22. Morozova, L. A., Haezebrouck, P., and Van Cauwelaert, F. (1991) Stability of equine lysozyme. 1. Thermal unfolding behavior. *Biophys. Chem. 41*, 185−191.
23. Van Dael, H., Haezebrouck, P., Morozova, L., Arico-Muendel, C., and Dobson, C. M. (1993) Partially folded states of equine lysozyme. Structural characterization and significance for protein folding. *Biochemistry 32*, 11886−11894.
24. Joniau, M., Haezebrouck, P., Noyelle K., and Van Dael, H. (2001) Structural basis for the appearance of a molten globule state in chimeric molecules derived from lysozyme and α-lactalbumin. *Proteins*: *Struct., Funct., Genet. 44*, 1−11.
25. Hendrix, T. M., Griko, Y. V., and Privalov, P. L. (1996) Energetics of structural domains in α-lactalbumin. *Protein Sci. 5*, 923−931.
26. Radford, S. E., Woolfson, D. N., Martin, S. R., Lowe, G., and Dobson, C. M. (1991) A three-disulphide derivative of hen lysozyme. Structure, dynamics, and stability. *Biochem. J. 273*, 211−217.
27. Peng, Z.-y., and Kim, P. S. (1994) A protein dissection study of a molten globule. *Biochemistry 30*, 3248−3252.
28. Baum, J., Dobson, C. M., Evans, P. A., and Hanely, C. (1989) Characterization of a partly folded protein by NMR methods: studies on the molten globule state of guinea pig α-lactalbumin. *Biochemistry 28*, 7−13.
29. Polverino de Laureto, P., Scaramella, E., Frigo, M., Gefter Wondrich, F., De Filippis, V., and Fontana A. (1999) Limited proteolysis of bovine α-lactalbumin: isolation and characterization of protein domains. *Protein Sci. 8*, 2290−2303.
30. Schulman, B. A., Kim, P. S., Dobson, C. M., and Redfield, C. (1997) A residue-specific NMR view of the noncooperative unfolding of a molten globule. *Nat. Struct. Biol. 4*, 630−634.
31. Schulman, B. A., Redfield, C., Peng, Z.-y., Dobson, C. M., and Kim, P. S. (1995) Different subdomains are most protected from hydrogen exchange in the molten globule and native states of human α-lactalbumin. *J. Mol. Biol. 253*, 651−657.
32. Demarest, S. J., Zhou, S.-Q., Robblee, J., Fairman, R., Chu, B., and Raleigh, D. P. (2001A) A comparative study of peptide models of the α-domain of α-lactalbumin, lysozyme, and α-lactalbumin/lysozyme chimeras allows the elucidation of critical factors that contribute to the ability to form stable partially folded states. *Biochemistry 40*, 2138−2147.
33. Demarest, S. J., Horng, J.-C., and Raleigh, D. P. (2001B) A protein dissection study demonstrates that two specific hydrophobic clusters play a key role in stabilizing the core structure of the molten globule state of human α-lactalbumin. *Proteins*: *Struct., Funct., Genet. 42*, 237−242.
34. Demarest, S. J., Fairman, R., and Raleigh, D. P. (1998) Peptide models of local and long-range interactions in the molten globule state of human α-lactalbumin. *J. Mol. Biol. 283*, 279−291.
35. Demarest, S. J., Boice, J. A, Fairman, R., and Raleigh, D. P. (1999) Defining the core structure of the α−lactalbumin molten globule state. *J. Mol. Biol. 294*, 213−221.
36. Kobashigawa, Y., Demura, M., Koshiba, T., Kumaki, Y., Kuwajima, K., and Nitta, K. (2000) Hydrogen exchange study of canine milk lysozyme: Stabilization mechanism of the molten globule. *Proteins 40*, 579−589.
37. Semisotnov, G. V., Rodionova, N. A., Razgulyaev, O. I., Uversky, V. N., Gripas, A. F., and Gilmanshin, R. I. (1991) Study of the "molten globule" intermediate state in protein folding by a hydrpophobic fluorescent probe. *Biopolymers 31*, 119−128.
38. Matullis, D., and Lovrien, R. (1998) 1-Anilino-8-naphthalene sulfonate anion-protein binding depends primarily on ion pair formation. *Biophys. J. 74*, 422−429.
39. Rohl, C. A., and Baldwin, R. L. (1997) Comparison of NH exchange and circular dichroism as techniques for measuring the parameters of helix−coil transition in peptides. *Biochemistry 36*, 8435−8442.
40. Horng, J.-C., Demarest, S., and Raleigh, D. P. (2003) pH-dependent stability of the human α-lactalbumin molten globule state: contrasting roles of the 6−120 disulfide and the *β*-subdomain at low and neutral pH. *Proteins*: *Struct., Funct., Genet. 52*, 193−202.
41. Kraulis, P. J. (1991) MOLSCRIPT: a program to produce both detailed and schematic plots of protein structures. *J. Appl. Crystallogr 24*, 946−950.
42. Acharya, K. R., Stuart, D. I., Walker, N. P. C., Lewis, M., and Phillips, D. C. (1989) Refined structure of baboon α-lactalbumin at 1.7 Å resolution. *J. Mol. Biol. 208*, 99−127.

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