Disulfide Determinants of Calcium-Induced Packing in α-Lactalbumin[†]

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ABSTRACT: α-Lactalbumin (α-LA) is a two-domain calcium-binding protein that folds through a molten globule intermediate. Calcium binding to the wild-type α-LA molten globule induces a transition to the native state. Here we assess the calcium-binding properties of the α-LA molten globule by studying two variants of α-LA. α-LA(α) contains only the two disulfide bonds in the α-helical domain of α-LA, while α-LA(β) contains only the β-sheet domain and interdomain disulfide bonds. We find that only α-LA(β) binds calcium, leading to the cooperative formation of substantial tertiary interactions. In addition, the β-sheet domain acquires a native-like backbone topology. Thus, specific interactions within α-LA imposed by the β-sheet domain and interdomain disulfide bonds, as opposed to the two α-helical domain disulfides, are necessary for the calcium-induced progression from the molten globule toward more native-like structure. Our results suggest that organization of the β-sheet domain, coupled with calcium binding, comprises the locking step in the folding of α-LA from the molten globule to the native state.

Native proteins are distinguished from partially folded forms by rigid side chain packing and extensive tertiary interactions. Many proteins fold through the molten globule, a compact but highly dynamic species with a native-like backbone topology (Kuwajima, 1989; Dobson, 1992; Ptitsyn, 1992, 1995). Native packing and tertiary interactions are subsequently acquired from the molten globule (Matthews, 1993), but our understanding of the structural and mechanistic bases of these events is poor.

α-Lactalbumin (α-LA)[1] is a widely studied calcium-binding protein composed of two structural domains. Folding of α-LA proceeds in two major steps (Ikeguchi et al., 1986; Kuwajima et al., 1990; Ptitsyn, 1992; Dobson et al., 1994; Peng & Kim, 1994; Wu et al., 1995). Collapse of the polypeptide chain yields a molten globule intermediate, which then acquires rigid side chain packing and tertiary interactions to form the native protein. We previously assessed the structure of the α-LA molten globule by studying the properties of two disulfide variants, α-LA(α) and α-LA(β) (Wu et al., 1995). Our studies indicated that the molten globule of α-LA is a bipartite structure in which the α-helical domain adopts a native-like backbone topology, while the β-sheet domain is largely unstructured (Peng & Kim, 1994; Wu et al., 1995).

Formation of the α-LA molten globule is not dependent on calcium (Ikeguchi et al., 1986; Kuwajima, 1989). In addition, the equilibrium molten globule of α-LA is studied in the absence of calcium [for reviews, see Kuwajima (1989)

and Ptitsyn (1992)]. Calcium binding to α-LA appears to induce native structure from the molten globule and affects the rate of folding from the molten globule to the native state (Dolgikh et al., 1981; Hiraoka & Sugai, 1984; Ikeguchi et al., 1986; Kuwajima et al., 1990; Ewbank & Creighton, 1993a,b). Furthermore, calorimetric and structural analyses of equine lysozyme, a calcium-binding lysozyme structurally homologous to α-LA, indicate that the protein unfolds in two stages, the first of which is calcium-dependent and results in the loss of specific tertiary interactions and side chain packing (Van Dael et al., 1993; Griko et al., 1995). Here we study the calcium-binding properties of α-LA(α) and α-LA(β), in order to localize the disulfide determinants of native packing and tertiary interactions in α-LA.

MATERIALS AND METHODS

Production of α-LA Variants. α-Lactalbumin variants were produced and purified as described previously from the expression plasmids pALA-A2 and pALA-B2 for α-LA(α) and α-LA(β), respectively (Wu et al., 1995).

Sedimentation Equilibrium. Sedimentation equilibrium experiments were performed on a Beckman XL-A analytical ultracentrifuge as described previously (Wu et al., 1995). Our data indicate that slight aggregation of α-LA(α) and α-LA(β) occurs at high concentrations of $CaCl_2$ (∼15% aggregation at 1 mM $CaCl_2$). Based on this observation and the results of the calcium titration (see below), a concentration of 200 μM $CaCl_2$ was used for all further studies of α-LA(α) and α-LA(β) in the presence of calcium. Protein solutions were dialyzed overnight against 10 mM Tris, pH 8.5, with either 0.5 mM EDTA or 200 μM $CaCl_2$. Initial protein concentrations of 100, 40, and 15 μM were analyzed at 23 and 27 krpm. The data for α-LA(α) and α-LA(β) in both the presence and absence of calcium fit well (within 8%) to a model for an ideal monomer, with no systematic deviation of the residuals. There is no concentration dependence of the observed molecular weight for either variant.

Circular Dichroism (CD) Spectroscopy. CD spectroscopy was performed with an Aviv 62 DS spectrometer as described

[1] Abbreviations: α-LA, α-lactalbumin; α-LA(α), human α-lactalbumin with cysteines 61, 73, 77, and 91 replaced by alanines, leaving the α-helical domain disulfide bonds intact; α-LA(β), human α-lactalbumin with cysteines 6, 28, 111, and 120 replaced by alanines, leaving the β-sheet domain and interdomain disulfide bonds intact; CD, circular dichroism; NMR, nuclear magnetic resonance; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GuHCl, guanidine hydrochloride; TMSP, (trimethylsilyl)propionate.
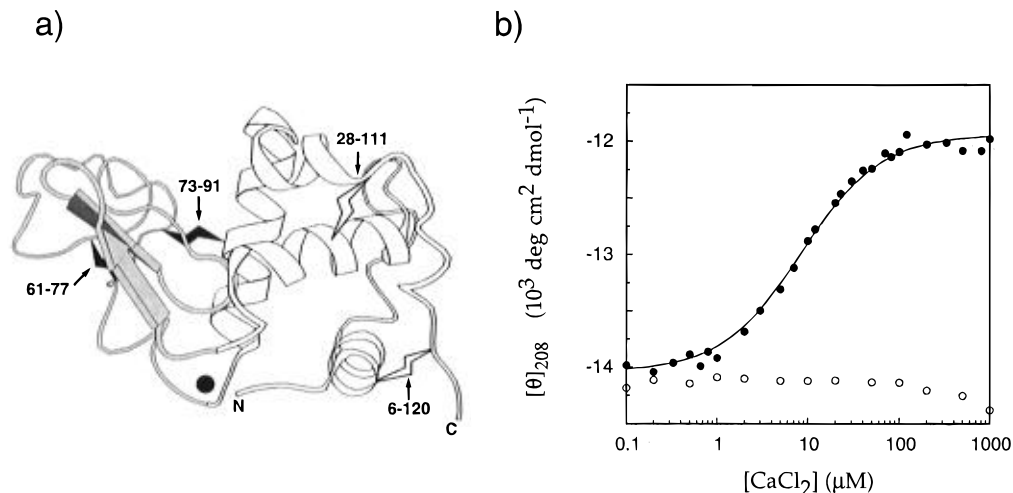
a)

b)

FIGURE 1: Calcium induces structural changes in α-LA(β), but not α-LA(α). (a) Schematic representation of human α-LA (Acharya et al., 1989, Acharya et al., 1991) produced with the program RIBBON (Priestle, 1988). The α-helical domain (white) contains the four α-helices. The β-sheet domain (shaded) contains two short β-strands and several loop structures. A single calcium ion (black ball) binds to the calcium-binding loop, comprised of residues 78−89. α-LA(α) contains the two disulfide bonds in the α-helical domain (6−120 and 28−111; white), with the β-sheet domain and interdomain cysteines replaced by alanines. α-LA(β) contains the disulfide bond in the β-sheet domain (61−77; black) and the interdomain disulfide bond (73−91; black), with the cysteines in the α-helical domain replaced by alanines. (b) Calcium titration of α-LA(α) (open circles) and α-LA(β) (filled circles) at 4 °C, pH 8.5, monitored by the CD signal at 208 nm.

previously (Wu et al., 1995). Samples were dissolved in 10 mM Tris, pH 8.5, with either 0.5 mM EDTA or 200 μM CaCl$_2$. The spectra of native α-LA were taken in 10 mM Tris, 1 mM CaCl$_2$, pH 8.5. Thermal denaturation data were collected at 208 nm at 2 °C intervals, allowing 1.5 min equilibration time and 60 s data averaging, using samples dissolved to a concentration of 2.5 μM in 10 mM Tris, pH 8.5, with either 0.5 mM EDTA or 200 μM CaCl$_2$. Denaturation curves were smoothed by least-squares fitting to a third-order polynomial, using a window of 10 data points. All thermal melts are >90% reversible with no hysteresis. Protein concentrations were determined by the absorbance in 6 M GuHCl, 20 mM sodium phosphate, pH 6.5, using an extinction coefficient at 280 nm of 22 430 (Edelhoch, 1967).

Calcium Titration. Changes in the far-UV CD signal of α-LA(α) and α-LA(β) upon addition of CaCl$_2$ were monitored at 208 nm. Samples were dissolved to a concentration of 4 μM in 10 mM Tris, pH 8.5, pretreated with a chelating agent (Chelex, Bio-Rad). CaCl$_2$ was added in small aliquots from 300 μM, 3 mM, 30 mM, 300 mM, and 3 M stocks, and the CD signal was normalized for volume changes. The titration data for α-LA(β) were fit to a model for a single binding site with a nonlinear least-squares fitting program (Kaleidagraph, Abelbeck Software) to yield the dissociation constant.

Disulfide Exchange. Disulfide exchange studies were performed at 4 °C as described previously (Wu et al., 1995). Native buffer consisted of 10 mM Tris, pH 8.5, with either 0.5 mM EDTA or 200 μM CaCl$_2$. Denaturing buffer consisted of 6 M GuHCl, 10 mM Tris, and 200 μM CaCl$_2$, pH 8.5.

Nuclear Magnetic Resonance (NMR) Spectroscopy. Protein samples were dissolved in D$_2$O to a concentration of ~100 μM at pH 8.5 (uncorrected for isotope effects), and either 0.5 mM deuterated EDTA or 200 μM CaCl$_2$. Both α-LA(α) and α-LA(β) are monomers under these conditions, as determined by sedimentation equilibrium. The spectrum of the pH 2 A-state molten globule of α-LA was acquired in D$_2$O adjusted to pH 2.0 with 0.1 M DCl (Peng & Kim,
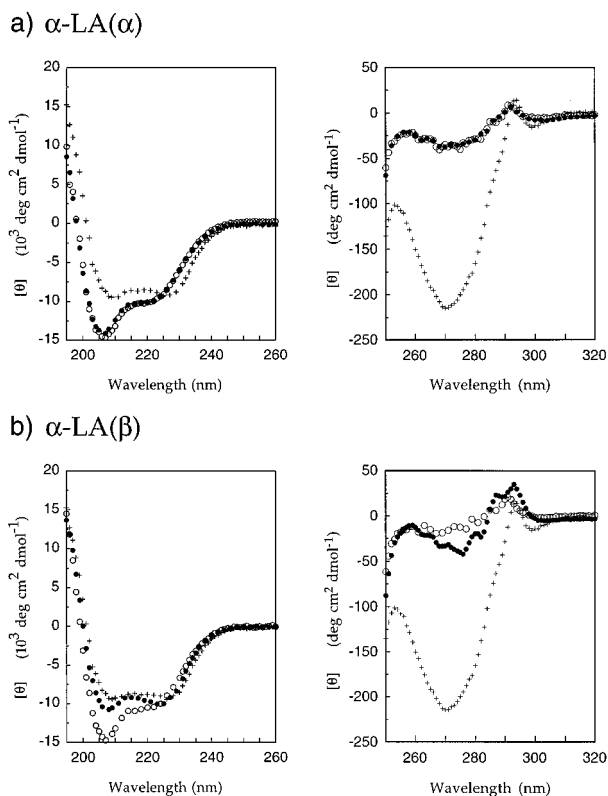
a) α-LA(α)

b) α-LA(β)

FIGURE 2: Far-UV and near-UV CD spectra of α-LA(α) and α-LA(β) in the presence (filled circles) and absence (open circles) of calcium at 4 °C, pH 8.5. Spectra of native α-LA (pluses) are also shown for comparison. (a) α-LA(α). (b) α-LA(β). The spectra of native α-LA and α-LA(α) and α-LA(β) in the absence of calcium have been published previously (Wu et al., 1995).

1994). ^{1}H NMR spectroscopy was performed at 500.1 MHz on a Bruker AMX spectrometer. 1D spectra were acquired at 4 °C using a spectral width of 7812.5 Hz, 4096 complex points, 32 768 transients, and a recycle delay of 1.5 s. The residual water peak was suppressed by mild presaturation. Chemical shifts were referenced to 0 ppm with internal (trimethylsilyl)propionate (TMSP).
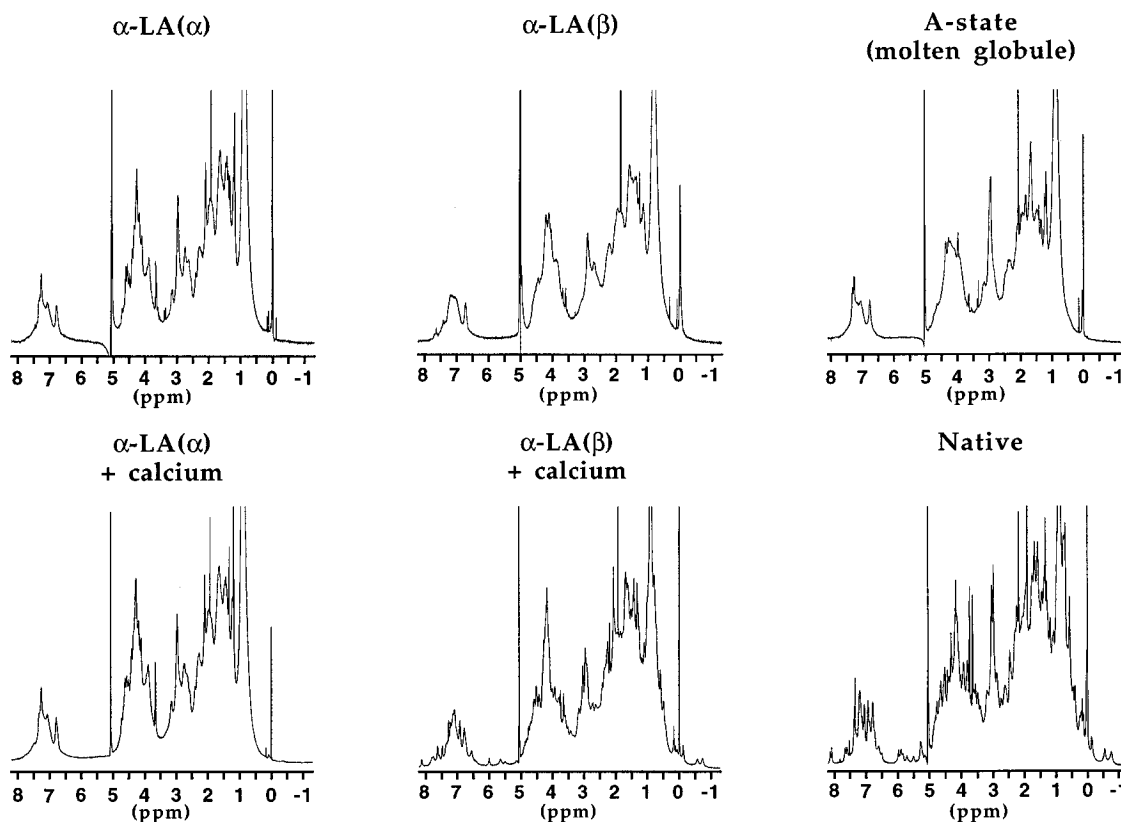
α-LA(α) α-LA(β) A-state
(molten globule)

α-LA(α)
+ calcium

α-LA(β)
+ calcium

Native

FIGURE 3: ^{1}H NMR spectra of α-LA(α) and α-LA(β) in the presence and absence of calcium at 4 °C, pH 8.5. For comparison, spectra of the pH 2 A-state and native α-LA (Peng & Kim, 1994) are also shown.

RESULTS

α-LA(α) corresponds to human α-LA, with the β-sheet domain and interdomain cysteines replaced by alanines, leaving the native 6−120 and 28−111 disulfide bonds intact (Figure 1a). Conversely, α-LA(β) corresponds to human α-LA, with the α-helical domain cysteines replaced by alanines, leaving the native 61−77 and 73−91 disulfide bonds intact. In the absence of calcium, α-LA(α) and α-LA(β) are both molten globules with structural properties very similar to the widely studied pH 2 molten globule (A-state) of α-LA (Wu et al., 1995).

Calcium does not affect the structural properties of α-LA(α), as judged by far-UV CD titrations of up to 1 mM calcium (Figure 1b). Indeed, the far-UV and near-UV CD spectra (Figure 2a) of α-LA(α) in the presence of calcium are superimposable on those of the calcium-free molten globule of α-LA(α). Moreover, the ^{1}H NMR spectra of α-LA(α) are identical in the presence and absence of calcium, and resemble closely that of the A-state molten globule of α-LA (Figure 3). Finally, disulfide exchange studies (Peng & Kim, 1994; Wu et al., 1995) under native conditions in both the presence and absence of calcium give identical results (data not shown).

On the other hand, α-LA(β) binds calcium with a K_d of $6.6 \pm 0.3 \ \mu M$ (Figure 1b). For comparison, the K_d of wild-type α-LA is 2−10 nM, depending on solution conditions[2] (Permyakov et al., 1981; Segawa & Sugai, 1983; Hamano et al., 1986; Mitani et al., 1986). The far-UV CD spectrum of calcium-bound α-LA(β) resembles closely that of native α-LA (Figure 2b). The near-UV CD of calcium-bound α-LA(β) is more intense than that of the α-LA(β) molten
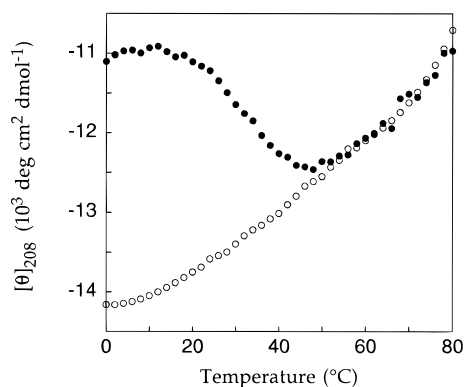
FIGURE 4: Thermal denaturation studies of α-LA(β) in the presence of calcium indicate that formation of the native-like structure is cooperative. CD signals of α-LA(β) in the presence (filled circles) and absence (open circles) of calcium are shown.

globule, indicative of tighter side chain packing, but it is substantially less intense than that of native α-LA (Figure 2b). These results suggest that, upon addition of calcium, α-LA(β) acquires more native-like structure, but does not become fully native. Thermal denaturation studies indicate that the formation of this structure is cooperative (Figure 4).

In the molten globule of α-LA, the β-sheet domain is largely unstructured, lacking a marked preference for native

[2] We determined the K_d of calcium binding to α-LA(β), a variant of human α-LA, in 10 mM Tris, pH 8.5, 0 °C. Reported K_d's for wild-type bovine α-LA are 1.7×10^{-9} M, measured in 5 mM Tris, pH 8.04, 20 °C (Permyakov et al., 1981), and 1×10^{-8} M, measured in 60 mM Tris, pH 8.4, 25 °C (Hamano et al., 1986). The K_d of human α-LA is ~20% greater than that of bovine α-LA under identical conditions (Segawa & Sugai, 1983).

a)

b)

c)
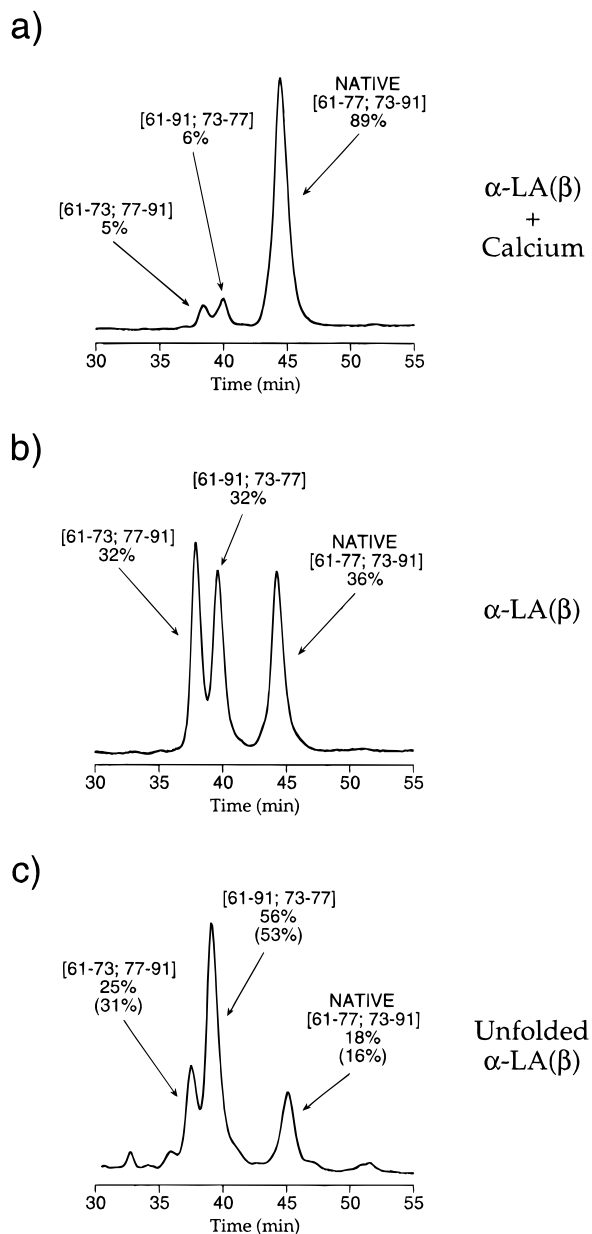

FIGURE 5: Disulfide exchange studies of α-LA(β) at 4 °C, pH 8.5. (a) Native conditions with calcium. (b) Native conditions without calcium (molten globule). (c) Denaturing conditions. The expected equilibrium populations (calculated with a random-walk model) for α-LA(β) under denaturing conditions are given in parentheses in (c). The numbers in brackets denote the disulfide-bonded residues in each species.

disulfide pairings in disulfide exchange assays, although the α-helical domain has a native-like tertiary fold (Peng & Kim, 1994; Wu et al., 1995). Under denaturing conditions (6 M GuHCl) in the presence of calcium, only 18% of the α-LA(β) molecules assume the native disulfide pairings, in agreement with the behavior predicted for an unfolded polypeptide, using a random-walk model (Kauzman, 1959; Snyder, 1987). Strikingly, addition of calcium to α-LA(β) under native conditions permits 90% of the α-LA(β) molecules to assume the native disulfide pairings (Figure 5a), in sharp contrast to the molten globule (Figure 5b) and unfolded (Figure 5c) forms of α-LA(β).

Although the CD and disulfide exchange studies indicate that calcium binding induces more native structure in the β-sheet domain, the lack of a significant increase in near-UV CD intensity in calcium-bound α-LA(β) suggests that the molecule is still flexible. The NMR spectrum of α-LA(β) in the absence of calcium is broad, lacks chemical shift dispersion, and resembles closely the ^{1}H NMR spectrum of the A-state molten globule of α-LA (Figure 3). However, the NMR spectrum of calcium-bound α-LA(β) (Figure 3) contains extensive chemical shift dispersion, including resonances shifted upfield of TMSP, indicative of substantial tertiary interactions. These features suggest that a significant amount of folded structure exists in calcium-bound α-LA(β), albeit less than in native α-LA.

DISCUSSION

Previous studies indicate that in the molten globule of α-LA, the α-helical domain is a dynamic, native-like structure, while the β-sheet domain is largely unstructured (Wu et al., 1995). Calcium induces the transition between the molten globule and the native state of α-LA. We find that calcium binding to α-LA(β) introduces specific structure, while α-LA(α) remains a molten globule in the presence of calcium. Thus, the β-sheet domain (61−77) and interdomain (73−91) disulfide bonds, as opposed to the α-helical domain disulfides, are crucial for the calcium-induced progression from the α-LA molten globule toward native structure.

The folding of lysozyme, a protein homologous to α-LA, proceeds via a kinetic molten globule intermediate in which the α-helical domain is native-like, while the β-sheet domain remains largely disordered (Radford et al., 1992; Miranker et al., 1993; Dobson et al., 1994; Balbach et al., 1995). Acquisition of both a near-UV CD signal and enzymatic activity are single kinetic events late in the folding pathway, with rates identical to the rate of folding of the β-sheet domain determined by hydrogen-exchange NMR (Radford et al., 1992; Dobson et al., 1994; Itzhaki et al., 1994).

Thus, in both α-LA and lysozyme, although the α-helical domain folds first, interactions outside of the α-helical domain are important for the acquisition of native packing and tertiary interactions. Our studies, taken together with previous work (Radford et al., 1992; Miranker et al., 1993; Dobson et al., 1994; Peng & Kim, 1994; Schulman et al., 1995; Wu et al., 1995), suggest the following pathway for the folding of the structurally homologous α-lactalbumins and lysozymes. Initial formation of the molten globule yields a species in which the α-helical domain is native-like, while the β-sheet domain is predominantly unfolded. Subsequently, a locking step requiring organization of the β-sheet domain, and calcium binding in α-LA, yields the unique native structure.

It is interesting that the NMR spectrum of calcium-bound α-LA(β) suggests significant tertiary contacts, while the near-UV (aromatic) CD spectrum lacks much of the intensity of native α-LA. Our studies indicate that the β-sheet domain has a native-like fold in calcium-bound α-LA(β). However, the detailed structure of calcium-bound α-LA(β) remains unclear. Many possibilities are apparent, in which the individual domains of α-LA have varying degrees of native structure.

At one extreme is the possibility that calcium binding to α-LA(β) yields slightly more structure throughout the entire molecule, converting α-LA(β) from a molten globule to a "highly ordered molten globule", a flexible, partially folded species in which the native secondary structures are largely formed, but loop regions are disordered (Feng et al., 1994;

Redfield et al., 1994). At the other extreme is the possibility that calcium binding to α-LA(β) causes the β-sheet domain to fold entirely, while the α-helical domain remains dynamic; the relatively small near-UV CD signal may result from the fact that only one of three tryptophans and one of four tyrosines in α-LA are present in the β-sheet domain. This second possibility is consistent with the previous identification of a stable two-disulfide species of α-LA involving the β-sheet domain and interdomain disulfide bonds, which is transiently populated during reduction of α-LA in the presence of calcium (Ewbank & Creighton, 1993a,b). High-resolution structural characterization or protein dissection of α-LA(β) in the presence of calcium should resolve this issue.

ACKNOWLEDGMENT

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