Local Structural Preferences in the α-Lactalbumin Molten Globule†

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ABSTRACT: Molten globules have been proposed to be general intermediates in protein folding. Despite numerous studies, a detailed description of the structure of a molten globule remains elusive. Recently, we showed that the molten globule formed by the helical domain of α-lactalbumin (α-LA) has a native-like backbone topology. Here we probe local structural preferences in the helical domain of the α-LA molten globule by analyzing a set of native and nonnative single disulfide bond variants using a combination of circular dichroism spectroscopy and determination of the equilibrium constant for disulfide bond formation. We find that the region surrounding the 28−111 disulfide bond has a high preference to adopt a native-like structure. Formation of other native or nonnative disulfide bonds is significantly less favorable. Our results suggest that molten globules contain regions with varying degrees of specificity for native-like structure and that the core region surrounding the 28−111 disulfide bond plays an important role in α-LA folding by stabilizing the molten globule intermediate.

Many proteins fold via molten globule intermediates that are characterized by compactness, near-native levels of secondary structure, an absence of rigid, specific side-chain packing, and a noncooperative thermal denaturation [for reviews, see Ptitsyn (1987), Kuwajima (1989), Christensen and Pain (1991), Ptitsyn (1992), and Haynie & Freire (1993)]. Classic molten globules, such as those formed by α-lactalbumin (α-LA),[1] carbonic anhydrase, and β-lactamase, have high conformational mobility and a low degree of side-chain ordering that preclude structure determination at atomic resolution.[2]

The best studied molten globule is that of α-LA (Kuwajima et al., 1976, 1985; Nozaka et al., 1978; Dolgikh et al., 1981, 1985; Ikeguchi et al., 1986; Baum et al., 1989; Ewbank & Creighton, 1991; Xie et al., 1991; Alexandrescu et al., 1993; Chyan et al., 1993; Creighton & Ewbank, 1994; Peng & Kim, 1994). α-LA is a two-domain protein, consisting of an α-helical domain and a β-sheet domain (Figure 1). Recently, we showed that a model of the isolated α-helical domain of α-LA (called α-Domain) forms a molten globule with a native-like tertiary fold (Peng & Kim, 1994). Subsequent studies show that the molten globule of intact α-LA has a bipartite structure. The α-helical domain adopts a native-like backbone topology, whereas the β-sheet domain remains largely unstructured (Wu et al., 1995).

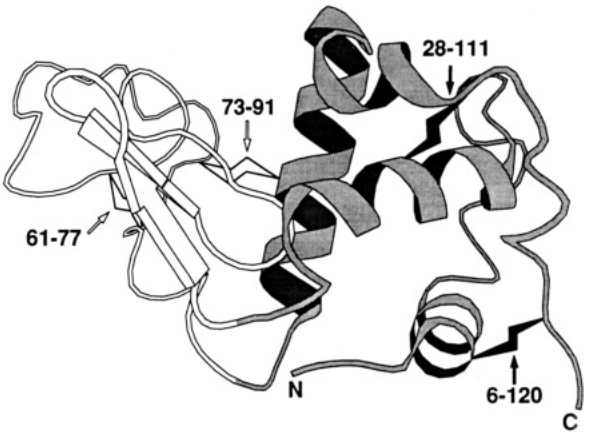

FIGURE 1: Schematic representation of α-LA (Priestle, 1988). The α-helical domain (residues 1−37 and 85−123) and the two disulfide bonds in the α-helical domain (28−111 and 6−120) are shaded (Acharya et al., 1989, 1991).

These results indicate that, even though it lacks extensive side chain packing interactions, the molten globule formed by the α-helical domain of α-LA retains much of the native protein's structural specificity (i.e., the ability of the polypeptide chain to distinguish the native structure from numerous nonnative structures). To investigate the origin of this specificity, we have produced six single-disulfide variants of α-LA, corresponding to all possible native and nonnative disulfide bonds in the α-helical domain. Each single-disulfide variant is characterized by circular dichroism (CD) spectroscopy and determination of the effective concentration (C_{eff}) for disulfide bond formation. C_{eff} is the ratio of equilibrium constants for intra- and intermolecular disulfide reactions and reflects the extent that specific interactions within the polypeptide chain favor the formation of a particular disulfide bond (Page & Jencks, 1971; Creighton, 1983; Lin & Kim, 1989, 1991). Thus, the structural specificity of local regions surrounding specific disulfide bonds can be quantitated and compared by C_{eff} measurements.

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[1] Abbreviations: α-LA, α-lactalbumin; [6−28], [6−111], [6−120], [28−111], [28−120], [111−120], single disulfide variants of human α-LA containing one disulfide bond between the cysteine residues indicated in the brackets, with all other cysteines replaced by alanines; CD, circular dichroism; C_{eff}, effective concentration; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; GuHCl, guanidine hydrochloride; GSH, reduced glutathione; GSSG, oxidized glutathione; $[\theta]_{222}$, mean residue ellipticity at 222 nm.

[2] Molten globules have not been crystallized successfully. The NMR spectra of classic molten globules are broad and lack chemical shift dispersion, inhibiting direct assignment. In contrast, "highly ordered molten globules" are partially folded species that yield high-resolution NMR spectra. The structures of two highly ordered molten globules have been solved recently (Feng et al., 1994; Redfield et al., 1994) and closely resemble that of the corresponding native protein.

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MATERIAL AND METHODS

Cloning, Protein Expression, and Purification. The gene for human α-LA was synthesized using the most frequently occurring *Escherichia coli* codons (Z.-y. Peng and P. S. Kim, unpublished results). The synthetic gene was cloned into the T7-polymerase-based expression vector pAED4 (Studier et al., 1990; Doering, 1992). The resulting plasmid is denoted pALA. Single disulfide variants of α-LA were produced by oligonucleotide-directed mutagenesis of the wild-type gene (Kunkel et al., 1987). Mutations were confirmed by dideoxynucleotide sequencing. The recombinant α-LA has an additional N-terminal methionine, as determined by amino acid sequencing and laser desorption mass spectrometry. Wild-type recombinant α-LA retains full activity in stimulating the galactose transfer reaction (Fitzgerald et al., 1970) and gives the same CD spectra as commercial human α-LA obtained from Sigma (Z.-y. Peng and P. S. Kim, unpublished results).

The single-disulfide variants of α-LA were expressed in *E. coli* BL21 (DE3) pLysS (Studier et al., 1990) and purified from inclusion bodies by ion exchange chromatography as described previously (Peng & Kim, 1994). Briefly, fractions containing an α-LA variant were combined, reduced by DTT, and dialyzed against 5% acetic acid. Reduced protein was purified from the dialyzed material by reversed-phase HPLC on a Vydac C_{18} column with an H_2O−acetonitrile gradient. Reduced, lyophilized protein was oxidized in 4 M GuHCl and 0.2 M Tris, pH 8.5, at room temperature for 48−72 h. The oxidized α-LA variant was purified further by reversed-phase HPLC.

Circular Dichroism and Equilibrium Sedimentation. CD studies were performed on an AVIV 62DS circular dichroism spectrometer equipped with a thermoelectric temperature controller. Far-UV CD spectra were taken at 0 °C in 10 mM Tris and 0.5 mM EDTA, pH 8.5 with 20 μM protein, a 1 mm path length cuvette, and a 1.5 nm bandwidth. For near-UV studies, 40 μM protein, a 1 cm path length cuvette, and a 5 nm bandwidth were used. Thermal denaturations were monitored at 222 nm in a 1 cm path length cuvette. All denaturation curves were greater than 90% reversible. Protein concentrations were determined by the absorbance at 280 nm in 6 M GuHCl (Edelhoch, 1967).

Equilibrium sedimentation was performed in a Beckman XL-A analytical ultracentrifuge. Protein solutions were dialyzed extensively against 10 mM Tris and 0.5 mM EDTA, pH 8.5. Three concentrations (~7, ~20, and ~60 μM) were analyzed at 4 °C, and the data were collected at three wavelengths (chosen between 230 and 285 nm) for two rotor speeds of 25 000 and 30 000 rpm. The apparent molecular weights were determined using the program NONLIN (courtesy of M. L. Johnson and J. Lary, University of Connecticut) with a partial specific volume of 0.729 for human α-LA (Laue et al., 1992).

Effective Concentration. Effective concentration (C_{eff}) measurements were performed in an anaerobic chamber (Coy Laboratory Products). Native buffer contains 10 mM Tris and 1 mM EDTA, pH 8.5; denaturing buffer contains 10 mM Tris, 1 mM EDTA, and 7.5 M GuHCl, pH 8.5. All buffers were degassed and stored under anaerobic conditions. Solutions of GSSG (sodium salt), GSH, and lyophilized proteins were made fresh in native buffer. The concentration of GSSG was determined spectroscopically at 248 nm using an extinction coefficient of 382 M^{-1} cm^{-1} (Huyghues-Despointes & Nelson, 1990). The concentration of GSH was determined by reaction with Ellman's reagent followed by measurement of the absorbance at 412 nm, using an extinction coefficient of 14 150 M^{-1} cm^{-1} (Ellman, 1959; Riddles et al., 1983). The C_{eff} measurements were initiated by diluting the protein stock solution to a 5 μM final concentration in redox buffer containing GSSG and GSH. The final reaction mix contains 10 mM Tris, 1 mM EDTA, and, for denaturing conditions, 6 M GuHCl. After equilibration periods of 7−8 and 22−24 h, aliquots of the sample were quenched by adding an equal volume of 10% acetic acid and were analyzed by reversed-phase HPLC. The amounts of oxidized and reduced proteins, which have the same extinction coefficients at 229 and 280 nm within experimental error (<5%), were determined by integrating the peak areas. For each variant, 4−6 independent measurements were made under at least two redox conditions. In each case, the results were the same within experimental error starting from either oxidized or reduced proteins, confirming that the reactions were at equilibrium.

RESULTS

We examined six variants of human α-LA that contain a single native or nonnative disulfide bond in the helical domain. All other cysteines, including those forming the disulfide bond in the β-sheet domain and the interdomain disulfide bond, were replaced by alanines. These variants are denoted by the cysteine residues that form the disulfide bond (e.g., [28−111]). In addition, as a reference protein, we made "all-Ala α-LA" in which all cysteines are replaced by alanines.

Sedimentation equilibrium studies indicate that all of the α-LA variants are monomers at concentrations between 7 and 60 μM at pH 8.5 with no additional salt. The apparent molecular weights of our α-LA variants measured at 7 and 20 μM were between 13.3K and 15.3K in close agreement with the expected molecular weight of the α-LA monomer (14.2K). At higher concentration (60 μM), the apparent molecular weight typically decreases by 8−15% due to the interaction with the counterion gradient (Williams et al., 1958). All subsequent experiments were carried out under these conditions.

Far-UV CD spectroscopy indicates that our single-disulfide variants of α-LA have different degrees of secondary structure (Figure 2A). As judged by near-UV CD spectroscopy, however, all variants lack the rigid, extensive side chain packing characteristic of native α-LA (Figure 2B). In addition, unlike native α-LA, all variants exhibit noncooperative thermal denaturation (data not shown).

We used far-UV CD spectroscopy to examine the effect on secondary structure of introducing native or nonnative disulfide bonds in the α-helical domain of α-LA (Figure 2A).[3] Only one variant, which contains the native 28−111 disulfide, exhibits a higher level of helix content than the

[3] Both aromatic side chains and disulfide bonds can produce CD signals in the far-UV region, although the contribution from disulfide bonds is usually small (Woody, 1985; Manning, 1989). The absence of substantial near-UV CD signals in our variants suggests that the aromatic and disulfide contribution to the far-UV CD signal will be minimal. We therefore assume that the dominant contribution to $[\theta]_{222}$ is from secondary structure.

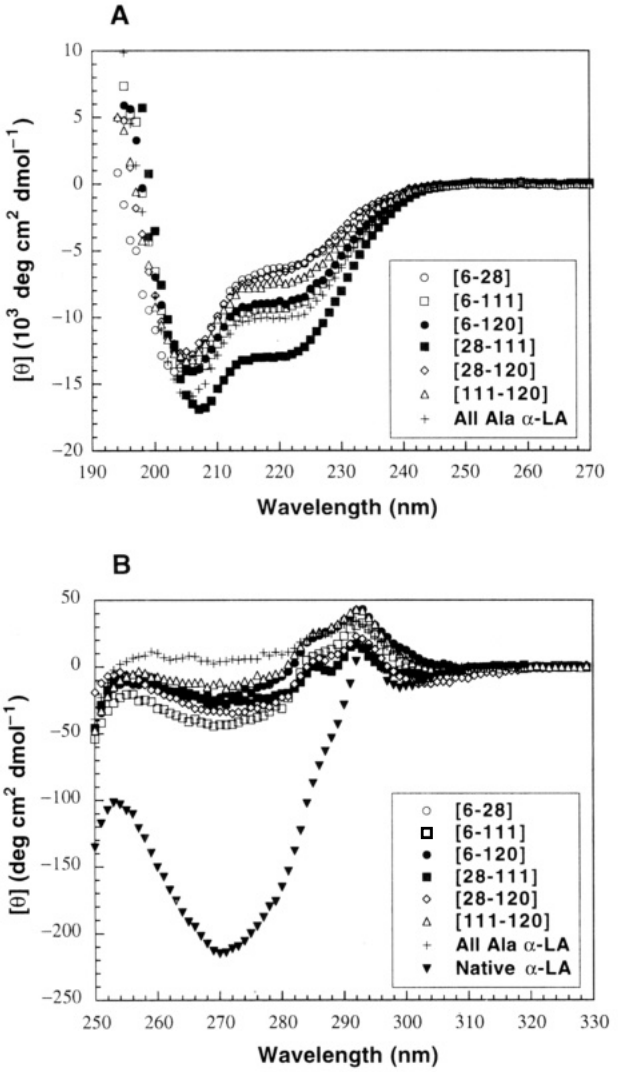

FIGURE 2: CD spectra of six single-disulfide variants. (A) Far-UV CD spectra of [6−28] (open circles), [6−111] (open squares), [6−120] (filled circles), [28−111] (filled squares), [28−120] (open diamonds), and [111−120] (open triangles). Filled symbols represent variants with a native disulfide, whereas open symbols represent variants with a nonnative disulfide. The reference for comparison of secondary structure is "all-Ala α-LA", an α-LA variant in which all cysteines are replaced by alanines (pluses). (B) Near-UV CD spectra of the same set of proteins. All variants show much weaker near-UV CD signals as compared to native α-LA (inverse filled triangles), indicating a disrupted side-chain packing.

all-Ala reference protein. Conversely, formation of a nonnative disulfide bond with Cys 28 is particularly disruptive to the secondary structure. These results suggest that the 28−111 disulfide bond is important for maintaining the structure of the α-LA molten globule. All other single-disulfide variants, including the variant with the native 6−120 disulfide bond, have levels of secondary structure below that of all-Ala α-LA. Curiously, the variant with the nonnative 6−111 disulfide exhibits a slightly higher $[\theta]_{222}$ than the variant with the native 6−120 disulfide, possibly because of strain associated with the 6−120 disulfide bond (see below).

In order to investigate the specificity of local regions for native-like structure, we measured the C_{eff} for disulfide bond formation, with glutathione as the reference thiol. Control measurements under strongly denaturing conditions (Table 1, third column) reflect the intrinsic probability for disulfide

Table 1: Effective Concentrations of Disulfide Bond Formation in the Helical Domain of α-LA[a]

α-LA variant	C_{eff} in native buffer (mM)	C_{eff} in denaturing buffer (mM)
[28−111]	$(1.07 \pm 0.11) \times 10^3$	0.94 ± 0.13
[6−120]	46.7 ± 4.1	0.54 ± 0.08
[6−28]	62.4 ± 3.3	9.1 ± 0.7
[6−111]	29.1 ± 4.0	0.48 ± 0.13
[28−120]	71.7 ± 1.7	0.90 ± 0.09
[111−120]	40.0 ± 1.7	23.1 ± 1.3

[a] Each data point consists of 4−6 independent measurements, taken after 7−8 h of equilibration, starting from both oxidized and reduced proteins, for at least two different redox conditions. The C_{eff}'s obtained after 22−24 h equilibration are typically 10−15% higher; otherwise, the pattern of C_{eff}'s remains the same.

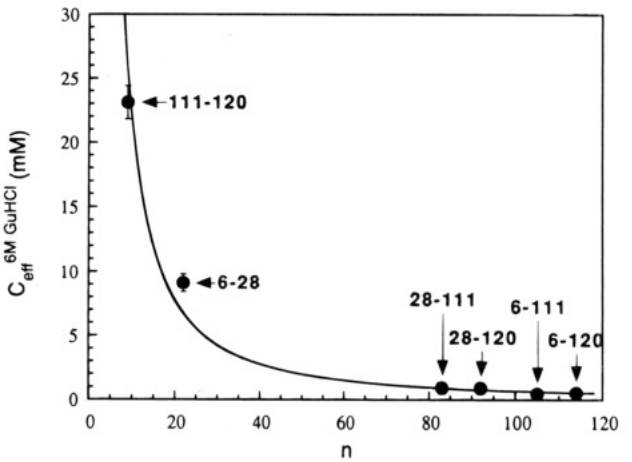

FIGURE 3: Effective concentration for formation of an intramolecular disulfide bond in the helical domain of α-LA under denaturing conditions, plotted as a function of the number of amino acid residues in the intervening loop. The line represents a single parameter fit to the random walk model of an unfolded polypeptide chain (Kauzmann, 1959), where $C_{eff} = an^{-3/2}$ and $n - 1$ is the number of non-cysteine residues in the loop. The best fit is obtained when $a = 694$ mM.

bond formation in the unfolded α-LA chain. These values agree well (Figure 3) with those predicted by the random walk theory of a unstructured polymer chain (Kauzmann, 1959), indicating that the Cys to Ala substitutions do not significantly perturb the unfolded state. Similar results have been observed previously for other unfolded proteins (Muthukrishnan & Nall, 1991).

Under native conditions, the C_{eff} for forming the native disulfide bond, 28−111, is at least an order of magnitude greater than the C_{eff} for forming any other native or nonnative disulfide bond (Table 1, second column). In contrast, the C_{eff} for forming the native 6−120 disulfide bond is in the same range as for forming a nonnative disulfide bond. This apparent lack of preference for forming the native 6−120 disulfide bond is consistent with the observation that the 6−120 disulfide bond is geometrically strained in the native α-LA structure and is hypersensitive toward reduction (Shechter et al., 1973; Kuwajima et al., 1990).

Remarkably, the structural specificity of the polypeptide chain is sufficiently high to allow Cys 28 to distinguish Cys 111 from Cys 120, even though these cysteines are separated by only nine amino acid residues. In addition, Cys 28 and Cys 111 are located far apart in the primary sequence, residing in different secondary structure elements and separated by the β-sheet domain. Thus, the high C_{eff} of 28−

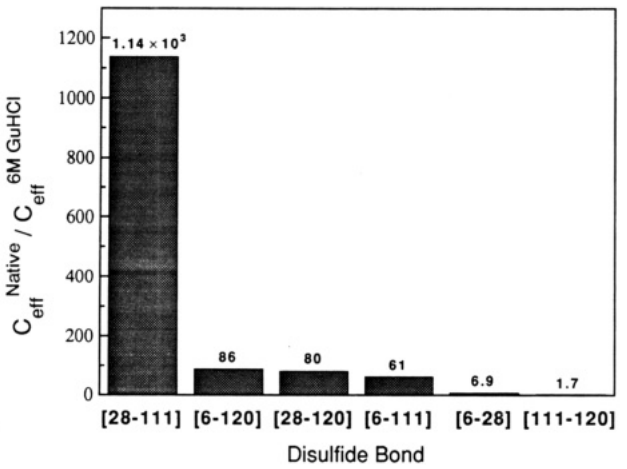

FIGURE 4: Ratio of C_{eff} between native and denaturing conditions (Lin & Kim, 1989) for each of the six possible disulfides in the helical domain of α-LA. The large ratio for the 28−111 disulfide bond suggests that the interactions between amino acid residues in the vicinity of this disulfide are largely responsible for stabilizing the molten globule of α-LA.

111 must result from interactions between residues that are distant in the sequence, rather than local structural propensities.

The ratio of C_{eff}'s under native and denaturing conditions gives, by thermodynamic linkage, the free energy of stabilization between the oxidized and reduced states (Creighton, 1983; Lin & Kim, 1989, 1991). Figure 4 shows the ratios for all six single-disulfide α-LA variants. The ratio for the native 28−111 disulfide bond is greater than 1000, strongly suggesting that this disulfide is stabilized by specific intramolecular interactions. On the other hand, the ratios for all other native and nonnative disulfide bonds are much smaller. The enhancement of the C_{eff} under native conditions (50−100-fold) for those disulfide bonds that are far apart in the primary sequence (6−120, 6−111, and 28−120) may result from the formation of a compact hydrophobically collapsed state, which brings distant cysteines closer together than in the unfolded random coil.

DISCUSSION

The striking result of our studies is that the C_{eff} for formation of the native 28−111 disulfide bond is more than 10 times higher than the C_{eff} for formation of any other native or nonnative disulfide bond. The α-LA variant with the 28−111 disulfide bond is the only variant with substantially more secondary structure than the all-Ala reference protein. On the other hand, all α-LA variants with nonnative disulfide bonds involving Cys 28 have significantly less secondary structure than all-Ala α-LA. Taken together, these results suggest that the local region surrounding the 28−111 disulfide bond has a high preference for adopting a native-like structure in the α-LA molten globule.

Unlike that of the native 28−111 disulfide, the C_{eff} for formation of the other native disulfide, 6−120, is within the same range as that of the nonnative disulfide bonds. This indicates that the 6−120 disulfide bond is not stabilized by specific interactions or that the stabilizing interactions are offset by unfavorable interactions such as disulfide bond strain. [The 6−120 disulfide bond can still have a nonspecific stabilization effect on the molten globule by reducing the chain entropy of the unfolded state; see Ikeguchi et al. (1992).] Thus, molten globules can be heterogeneous, containing regions which have varying degrees of specificity toward native-like structure.

A rough quantitation of the local structural specificity around the 28−111 disulfide bond in the α-LA molten globule can be made. The C_{eff}'s for each of the non-native disulfide bonds in the helical domain of α-LA fall within a narrow range (50 ± 20 mM), which can be taken as a representative C_{eff} value in the nonspecific hydrophobically collapsed protein. The C_{eff} for forming the native 28−111 disulfide bond is, however, 20 times higher than the average value for forming a nonnative disulfide bond. Assuming, to a first approximation, that the ratio of C_{eff} between native and nonnative disulfide bonds reflects the difference in free energy between native and nonnative structures, our results suggest that formation of the native-like structure around the 28−111 disulfide bond contributes ~2 kcal/mol of free energy to the stability of the molten globule relative to species with nonnative structures.

The single-disulfide variant of α-LA with the native 28−111 disulfide bond (denoted α-LA[28−111]) is likely to be a useful model system for future studies of molten globules. Previous studies of the α-LA molten globule are complicated by the presence of multiple disulfide isomers and disulfide exchange reactions. α-LA[28−111] exhibits the characteristics of a molten globule under a broad range of conditions, including near neutral pH. The single disulfide bond simplifies protein production and folding and can be used as a reporter of backbone topology in C_{eff} studies.

Two observations may explain why the 28−111 disulfide bond has a significantly higher C_{eff} than other disulfide bonds. First, the 28−111 disulfide bond is encompassed by regions that have a high content of helical secondary structure (helices B, C, and D are all nearby). Second, this disulfide is buried and is located near many hydrophobic residues. Our results suggest that hydrophobic interactions between regions with high local secondary structure propensities may play an important role in protein folding, even in the absence of extensive side chain packing interactions, by stabilizing molten globule intermediates.

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