Article No. mb981825 *J. Mol. Biol.* (1998) **280**, 175–182

JMB

A Specific Hydrophobic Core in the α-Lactalbumin Molten Globule

Lawren C. Wu and Peter S. Kim*

Howard Hughes Medical Institute, Whitehead Institute for Biomedical Research Massachusetts Institute of Technology, Department of Biology, Nine Cambridge Center, Cambridge, MA 02142 USA

*Corresponding author

Molten globules are partially structured protein folding intermediates that adopt a native-like overall backbone topology in the absence of extensive detectable tertiary interactions. It is important to determine the extent of specific tertiary structure present in molten globules and to understand the role of specific side-chain packing in stabilizing and specifying molten-globule structure. Previous studies indicate that a small degree of specific side-chain packing stabilizes the structures of the cytochrome *c*, apomyoglobin, and staphylococcal nuclease molten globules. Here we investigate the extent of specific side-chain packing in the molten globule of α-lactalbumin (α-LA), a highly fluctuating, non-cooperatively formed molten globule. By analyzing a set of point mutations in the helical domain of α-LA, we have identified a stabilizing hydrophobic core. Moreover, this core corresponds to a previously identified structural subdomain and likely contains some native-like packing interactions. Our results suggest that native-like packing of core amino acids helps stabilize molten globules and that some specific interactions can exist in even highly dynamic, fluctuating species.

Keywords: α-lactalbumin; molten globule; protein folding; disulfide bond; effective concentration

Introduction

Molten globules are compact, partially structured forms of proteins thought to be general intermediates in protein folding (Dobson, 1992; Kuwajima, 1989; Ptitsyn, 1992). Molten globules have high levels of native-like secondary structure arranged in an overall native-like fold (Peng *et al.*, 1995a), but lack rigid side-chain packing and extensive detectable tertiary interactions. As such, molten globules can be viewed as a ''low-resolution solution'' to the protein folding problem, in which a native-like protein architecture has been established. It is important to understand how an overall native-like fold can be formed in the apparent absence of extensive specific tertiary interactions. One extreme possibility is that molten globule structure is formed by non-specific hydrophobic collapse around a core of dynamic, loosely interacting residues. In this case, specific packing interactions may be minimal or non-existent, and molten globule structure may be determined by the global distribution of hydrophobic and hydrophilic amino acids, as opposed to specific amino acid identity. Another extreme possibility is that undetected, highly specific tertiary interactions stabilize molten globules. In this case, specific interactions amongst a small subset of key residues may be obscured by high conformational mobility in the remainder of the molecule.

The extent of specific native packing that exists in the molten globules of apomyoglobin, cytochrome *c*, and staphylococcal nuclease has been assessed by studying point mutants of hydrophobic core residues (Carra *et al.*, 1994; Colon & Roder, 1996; Colon *et al.*, 1996; Kay & Baldwin, 1996; Lin *et al.*, 1994; Marmorino & Pielak, 1995). In these studies, the effects of mutations on the stabilities of the molten globule states are small, suggesting that tertiary interactions are only partially formed in the molten globule. Nonetheless, the effects of point mutations on the stabilities of the native and molten globule states are correlated. These results suggest that a small degree of specific native-like packing stabilizes and helps determine the structure of the molten globule.

Abbreviations used: α-LA, α-lactalbumin; CD, circular dichroism.

0022–2836/98/260175–08 $30.00/0

The molten globule folding intermediate of α-lactalbumin (α-LA), a two domain protein containing four disulfide bonds, consists of a native-like helical domain and a largely unstructured β-sheet domain (Alexandrescu *et al.*, 1993; Kuwajima, 1996; Schulman *et al.*, 1995; Wu *et al.*, 1995). Molten globules can range in orderliness from highly dynamic species with poor NMR chemical shift dispersion and non-cooperatively formed structure, to highly ordered species with substantial NMR chemical shift dispersion and cooperatively formed structure (Alexandrescu *et al.*, 1993; Feng *et al.*, 1994; Redfield *et al.*, 1994). The α-LA molten globule is highly dynamic, yielding NMR spectra with broad linewidths and poor chemical shift dispersion. Moreover, formation of the α-LA molten globule is largely non-cooperative, as judged by proline scanning mutagenesis and denaturation transitions monitored at global and residue-specific levels (Schulman & Kim, 1996; Schulman *et al.*, 1997; Shimizu *et al.*, 1993). Furthermore, thermal denaturation of the α-LA molten globule is accompanied by little excess heat absorption, suggesting that the core of the molten globule may be loosely ordered and solvent exposed, although this is in debate (Pfeil *et al.*, 1986; Xie *et al.*, 1991; Yutani *et al.*, 1992). The extent of specific packing interactions in such a dynamic fluctuating species is unclear, but may be elucidated by systematic mutagenesis in the core of the molten globule.

There are two hydrophobic cores in the structure of native α-LA (Figure 1; Acharya *et al.*, 1991). One, called the hydrophobic box, comprises residues from the C and D-helices and the β-sheet domain (Acharya *et al.*, 1991). Another comprises residues from the A, B, and 3_{10}-helices. Measurements of the equilibrium constants for formation of native and non-native disulfide bonds in the helical domain of the α-LA molten globule indicate that the region around the 28–111 disulfide bond plays an important stabilizing role (Peng *et al.*, 1995b). Although the 28-111 disulfide bond connects the B and D-helices, which lie near the hydrophobic box, no direct evidence indicates that the hydrophobic box forms the stabilizing core of the molten globule. On the other hand, NMR studies delineate a stable structural subdomain in the α-LA molten globule, comprising the A, B, and 3_{10}-helices (Schulman *et al.*, 1997).

Here we analyze a collection of point mutants in the helical domain of α-LA, using the equilibrium constant for formation of the 28–111 disulfide bond to monitor effects on the stability of the molten globule. We find that residues from the A/B/3_{10} subdomain, as opposed to the hydrophobic box, form the major stabilizing hydrophobic core. Moreover, this core likely contains some specific native-like packing interactions that may help specify the native-like structure of the α-LA molten globule.

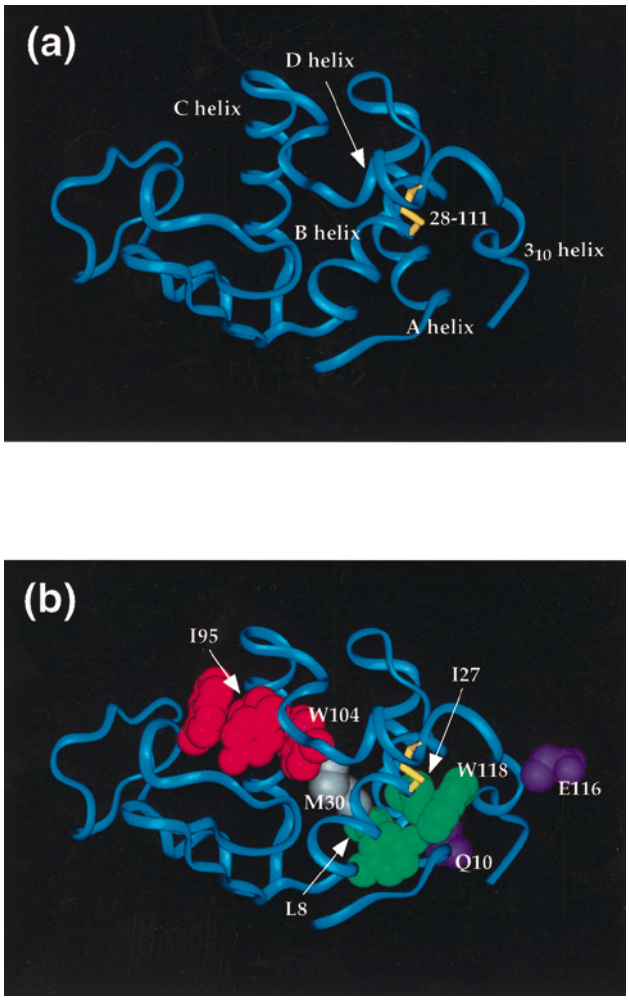

Figure 1. (a) Schematic representation of the structure of α-lactalbumin showing the polypeptide backbone and the 28–111 disulfide bond. The five major helices in the helical domain are indicated. (b) Depiction of the hydrophobic cores in α-LA and the wild-type residues that are mutated in this study. The molecule is oriented as in (a) with the side-chains comprising the hydrophobic box shown in red (I95 and W104 are mutated in this study), the side-chains comprising the A/B/3_{10} subdomain shown in green (L8, I27, and W118 are mutated in this study), and M30, which bridges the two hydrophobic cores, is shown in gray. The surface residues that are mutated in this study (Q10 and E116) are shown in purple.

Results

Mutagenesis scheme

We examined a collection of point mutations in and around the hydrophobic cores of α-LA, using the native structure of α-LA as a guide to probe the molten globule state (Figure 1). One hydrophobic core, the hydrophobic box, contains residues from the C and D-helices of the helical domain and parts of the β-sheet domain. Two central residues in the hydrophobic box are I95 and W104, which lie in the C-helix and just proxi-

mal to the D-helix, respectively. Both residues are fully buried and make extensive contact with each other in the native structure. The other hydrophobic core in α-LA consists of residues from the A, B, and 3_{10}-helices, associated with the A/B/3_{10} subdomain. Three central residues in this core are L8, I27, and W118, which lie in the A, B, and 3_{10}-helices, respectively. Thus, we examined a series of mutants at these and other nearby positions (Q10, M30, and E116). Our reference molecule is α-LA_{28-111}, a single disulfide variant of α-LA containing only the 28–111 disulfide bond, with all other cysteine residues changed to alanine. This variant has been characterized previously and forms a molten globule with properties similar to that of the wild-type α-LA molten globule with all four disulfide bonds (Peng *et al.*, 1995b; Schulman & Kim, 1996).

Overall secondary structure

Since molten globules are prone to aggregation, we determined the oligomerization state of α-LA_{28-111} and its variants using sedimentation equilibrium. Under native buffer conditions, all species studied here are monomeric (Table 1). Therefore, the effects of mutations in α-LA_{28-111} do not result from aggregation. We examined the overall secondary structure of α-LA_{28-111} mutants by measuring and comparing far-UV CD spectra. All mutants of α-LA_{28-111} have substantial helix content (Figure 2). Some variants have small decreases in helix content as compared to the α-LA_{28-111} reference, indicated by decreases in the magnitude of the CD signal at 222 nm (Table 1). Since the α-LA molten globule is formed relatively non-cooperatively, it is unclear whether the decreased helix content results from global destabilization of the molecule or local helix unfolding. Proline scanning mutagenesis and NMR studies indicate that single helices in the α-LA molten globule can unfold independently of structure in the remainder of the molecule (Schulman & Kim, 1996; Schulman *et al.*, 1997). The point mutations examined here may have similar effects.

Table 1. Structural characterization of α-LA_{28-111} and point mutants

α-LA_{28-111} variant	Oligomerization state[a]	$-[\theta]_{222}$ (deg cm^2 dmol^{-1})
Wild-type	1.02	13,000
L8F	0.98	12,200
L8I	1.02	10,900
L8A	1.03	10,100
I27W	1.06	11,200
I27F	0.96	10,700
I27L	0.96	13,000
I27A	0.95	11,800
W118L	0.96	11,100
W118A	0.95	12,700
I27A + W118A	1.03	10,800
Q10A	1.00	12,200
E116A	0.97	13,100
M30F	1.07	12,600
M30A	0.98	12,100
W104A	1.02	13,000
I95F	0.98	12,400
I95A	1.03	12,500

[a] Oligomerization state is determined by sedimentation equilibrium and is denoted as the ratio of the observed to the expected molecular weight.

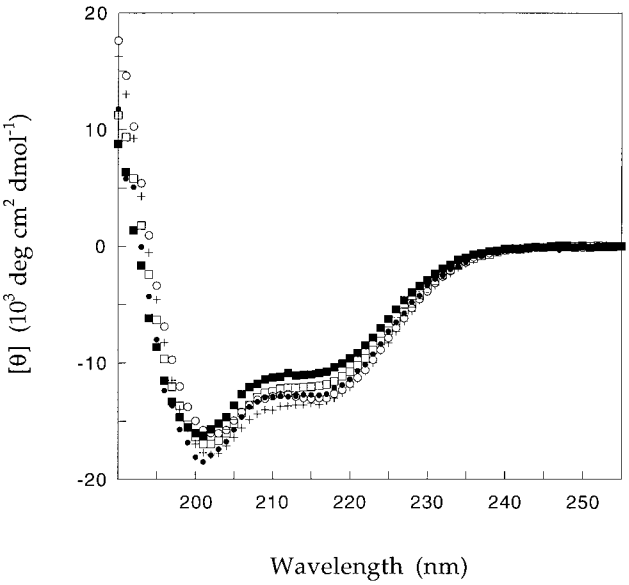

Figure 2. Circular dichroism spectra of wild-type α-LA_{28-111} and selected point mutants indicate that all molecules have substantial helix content, although some mutations cause a small decrease in overall helix content. Shown are α-LA_{28-111} (plus signs), W104A (open circles), W118A (filled circles), I27A (open squares), and the double mutant I27A+W118A (filled squares). W104 is in the hydrophobic box, while I27 and W118 are in the A/B/3_{10} subdomain.

Effective concentration delineated core structure

We investigated the effects of mutations on the stability of the α-LA molten globule by measuring the effective concentration of the 28–111 disulfide bond (C_{eff}), using glutathione as a reference thiol. C_{eff} is the ratio of equilibrium constants for intra and inter-molecular disulfide reactions and reflects the extent that specific interactions in the polypeptide chain favor formation of the disulfide bond (Creighton, 1983; Lin & Kim, 1989, 1991; Page & Jencks, 1971). Previous studies indicate that structure in the α-LA molten globule significantly enhances the C_{eff} of 28–111 (Peng *et al.*, 1995b). Thus, we assessed the effects of mutations in the α-LA molten globule by comparing C_{eff} measurements. It is important to establish that effects on the C_{eff} of 28–111 due to mutations do not arise from changes in the unfolded state. Control experiments carried out under denaturing conditions indicate that the C_{eff} of the 28–111 disulfide bond

is unchanged in the unfolded state, regardless of mutations (Table 2).

Under native conditions, mutations in the hydrophobic cores of the α-LA molten globule have markedly different effects (Table 2). Mutations of I95 and W104 in the hydrophobic box cause small decreases (up to 20%) in the C_{eff} of 28–111, despite significant changes in side-chain size and hydrophobicity. In contrast, mutations of residues L8, I27, and W118 in the $A/B/3_{10}$ subdomain can cause substantial decreases in the C_{eff} of 28–111 (up to 80%). Importantly, mutations of surface residues Q10 and E116, which lie on the outside of the $A/B/3_{10}$ subdomain (A and 3_{10}-helices, respectively), do not destabilize the molten globule. Thus, the $A/B/3_{10}$ core plays the major role in stabilizing the α-LA molten globule, while the hydrophobic box may be largely unstructured. Moreover, residues that are buried in the native structure, as opposed to residues on the surface, stabilize molten globule structure. Interestingly, mutations of M30, a fully buried B-helix residue that makes contacts with both the hydrophobic box and the $A/B/3_{10}$ subdomain in the native α-LA structure, cause significant decreases in the C_{eff} of 28–111, suggesting that M30 is associated with the $A/B/3_{10}$ subdomain in the molten globule.

Specific packing interactions

We assessed the specificity of packing interactions in the $A/B/3_{10}$ core by systematically mutating L8, I27, M30, and W118 to larger, smaller, and similarly sized hydrophobic amino acids. If the $A/B/3_{10}$ core consists of highly dynamic, loose hydrophobic interactions, then the core might be expected to accommodate considerable structural variation. Instead, neither larger nor smaller hydrophobic amino acids are well tolerated at residue I27, causing substantial decreases in the C_{eff} of 28–111. Moreover, even a relatively conservative mutation of I27 to leucine causes a significant decrease (28%) in the C_{eff} of 28–111. Similar effects are observed at residues L8 and M30, where neither larger, smaller, nor similarly sized amino acids are well accommodated. Interestingly, for a series of mutants at a given site, changes in helix content (Table 1) are roughly correlated with destabilizing effects assessed by the effective concentration for formation of the 28–111 disulfide bond. Thus, only wild-type residues comprising the $A/B/3_{10}$ hydrophobic core are well tolerated by the molten globule structure, suggesting that the core of the α-LA molten globule contains some specific native-like packing interactions. Notably, mutation of I95 in the hydrophobic box to either larger or smaller amino acids has negligible effects on the stability of the α-LA molten globule. The I95F mutation may even slightly stabilize the molten globule. These effects are consistent with an unstructured or loosely organized hydrophobic box.

Table 2. Effective concentrations of disulfide bond formation in α-$LA_{28\text{-}111}$

α-$LA_{28\text{-}111}$ variant	C_{eff} in native buffer (mM)	C_{eff} in denaturing buffer (mM)
Wild-type	1100 ± 80	1.09 ± 0.08
L8F	740 ± 100	1.13 ± 0.07
L8I	990 ± 80	1.07 ± 0.05
L8A	530 ± 20	1.11 ± 0.06
I27W	420 ± 30	1.07 ± 0.01
I27F	580 ± 50	1.01 ± 0.03
I27L	790 ± 50	1.01 ± 0.08
I27A	210 ± 20	1.01 ± 0.02
W118L	650 ± 30	0.93 ± 0.04
W118A	560 ± 30	1.11 ± 0.04
I27A + W118A	180 ± 10	0.95 ± 0.02
Q10A	1130 ± 80	1.06 ± 0.08
E116A	1380 ± 100	1.11 ± 0.06
M30F	850 ± 70	1.19 ± 0.05
M30A	630 ± 30	0.94 ± 0.04
W104A	940 ± 40	1.01 ± 0.13
I95F	1230 ± 90	1.06 ± 0.11
I95A	1100 ± 50	0.97 ± 0.13

Discussion

Hydrophobic core location

We have investigated the hydrophobic core structure of the α-LA molten globule by systematic mutagenesis of specific amino acids, using the C_{eff} of the 28–111 disulfide bond to assess effects on stability. Of the two hydrophobic cores in the helical domain of α-LA, the one associated with the $A/B/3_{10}$ subdomain plays the predominant role in stabilizing the molten globule. Mutations of residues in this core cause substantial decreases in the C_{eff} of the 28–111 disulfide bond. In contrast, mutations in the hydrophobic box have little or no impact on the C_{eff} of 28–111. Importantly, none of the side-chains studied here, except W118, directly contacts the 28–111 disulfide bond in native α-LA. Thus, the effects measured here likely arise from stabilization of the molten globule, as opposed to direct interaction with the disulfide bond.

Our results are consistent with other studies that assess core structure in the molten globules of α-LA and lysozyme, a protein homologous to α-LA. In one study, alanine scanning mutagenesis of all hydrophobic residues in the helical domain of the α-LA molten globule, using C_{eff} of the 28–111 disulfide as a measure of stability, describes a core structure similar to that deduced here (Song *et al.*, 1998). In other studies, NMR measurements of backbone amide hydrogen exchange in lysozyme molten globules delineate protected native-like hydrophobic cores similar to that observed here (Hooke *et al.*, 1994; Morozova *et al.*, 1995). Our results complement a residue-specific NMR study of the denaturation of the wild-type α-LA molten globule (with all four disulfide bonds; Schulman *et al.*, 1997). In this study, the regions of structure most resistant to unfolding correspond to the $A/B/3_{10}$ subdomain and encompass L8, I27,

M30, and W118, the residues shown here to play a substantial role in stabilizing the α-LA molten globule. In contrast, residues I95 and W104 (i.e. the hydrophobic box) are not part of the structural subdomain defined by NMR, since they unfold at lower concentrations of denaturant prior to disruption of the A/B/3_{10} subdomain. We find that I95 and W104 tolerate a wide variety of amino acid substitutions with little or no effect on the stability of the α-LA molten globule. Taken together, the NMR studies and our C_{eff} studies indicate that the hydrophobic box is poorly formed in the α-LA molten globule, resulting in loosely associated and marginally stable structure.

Native-like topology of the molten globule

Our mutagenesis is based on the native structure of α-LA and involves residues from distant parts of the polypeptide chain brought together by the tertiary fold. It is striking that our results are consistent with this structural model. This extends to the observation that residues in the native structure of α-LA, found buried in the core as opposed to on the surface, appear to play the dominant stabilizing role in the molten globule, as has been observed in native proteins (Bowie *et al.*, 1990; Matthews, 1995). Previous studies indicate that the helical domain in the α-LA molten globule contains native-like helices arranged in a native-like fold (Peng & Kim, 1994; Schulman *et al.*, 1995; Wu *et al.*, 1995). Our results provide additional evidence that molten globules have a native-like fold.

Specific native-like packing

Our results suggest that some specific native-like packing exists in the core of the α-LA molten globule. Neither larger nor smaller residues are well-tolerated in the A/B/3_{10} core. Moreover, even conservative amino acid mutations cause small but measurable destabilization of the molten globule. Specific native-like packing has also been inferred from studies of the molten globules of apomyoglobin, cytochrome *c*, and staphylococcal nuclease (Carra *et al.*, 1994; Colon & Roder, 1996; Colon *et al.*, 1996; Kay & Baldwin, 1996; Lin *et al.*, 1994; Marmorino & Pielak, 1995). Significantly, the α-LA molten globule is the most dynamic of these species, showing evidence for highly fluctuating structure and non-cooperative folding. It is interesting that specific native-like packing and a native-like fold can exist in even a highly fluctuating molten globule such as that of α-LA, which may correspond to an early folding intermediate in which hydrophobic collapse, but not formation of extensive fixed tertiary structure, has occurred. It is likely that the small amount of specific core packing associated with the A/B/3_{10} subdomain is obscured by highly mobile structure in the remainder of the molecule. In this regard, our studies are consistent with structural models of molten globules in which secondary structure comprising the core scaffold of the protein is formed in the molten globule state, while loop and peripheral regions are flexible and disordered (Kuwajima, 1989; Ptitsyn, 1992).

Comparison with a minimized α-LA sequence

Despite the existence of some specific packing in the core of the α-LA molten globule, we have demonstrated previously that all of the hydrophobic amino acids in the helical domain can be simultaneously replaced with leucine (the resulting construct is called MinLeu) without significantly affecting the overall fold at a low resolution (Wu & Kim, 1997). Based on our measurements here, simultaneous substitution of all hydrophobic amino acids with leucine might be expected to decrease the stability of the helical domain. I27 and W118, the two positions studied here that are amongst the 31 that are simultaneously changed to leucine in MinLeu, yield decreases in C_{eff} of 28–111 when changed to leucine in α-$LA_{28\text{-}111}$. However, we find that under native buffer conditions the effective concentration of the 28-111 disulfide bond in MinLeu is higher than that in the wild-type α-LA molten globule (3.67(±0.24) M and 1.10(±0.08) M, respectively).

Several explanations for the higher effective concentration are possible. Here, we have performed mutagenesis on only a subset of all hydrophobic residues in the helical domain. It is possible that replacement of other hydrophobic residues with leucine stabilizes the helical domain, either by removing destabilizing interactions (e.g. removing a buried polar interaction; cf. Lumb & Kim, 1995) or by introducing stabilizing interactions (e.g. increasing the helix propensity of an amino acid or introducing favorable side-chain interactions). Indeed, substitution of some residues in the helical domain of α-LA with more hydrophobic amino acids results in stabilization of the molten globule (e.g. I95F in this study; T29I, A30I, and T33I by Uchiyama *et al.*, 1995).

It is also possible that simultaneous substitution of multiple residues with leucine allows new side-chain conformations to be adopted that are not favorable individually. Tolerance of multiple amino acid substitutions that are unfavorable individually has been observed in repacked cores of several proteins (e.g. Lim & Sauer, 1989; Matthews, 1995). Finally, the polypeptide backbone in MinLeu may be arranged slightly differently than in wild-type α-LA, yielding a structure that is still native-like at low resolution but has a higher effective concentration for the 28–111 disulfide bond. Further experiments are needed to clarify how the 28–111 disulfide bond in MinLeu retains a high effective concentration.

Specific packing in protein folding

Our results strongly suggest that some specific side-chain packing exists in the α-LA molten

globule. Specific side-chain packing has also been inferred in the molten globules of other proteins, suggesting that early folding intermediates characterized by high conformational flexibility may still have cores containing some specific packing interactions (Carra *et al.*, 1994; Colon & Roder, 1996; Colon *et al.*, 1996; Kay & Baldwin, 1996; Lin *et al.*, 1994; Marmorino & Pielak, 1995). However, it is unclear whether specific side-chain packing, although present, is necessary for a native-like overall fold in molten globules.

Hydrophobic sequence minimization of the α-LA molten globule suggests that non-specific hydrophobic interactions, as opposed to specific packing interactions, are sufficient for the formation of a native-like backbone topology (Wu & Kim, 1997). In addition, substitution of amino acids in the core of α-LA with more hydrophobic amino acids may increase the stability of the molten globule, suggesting that hydrophobic interactions distinct from native-like packing may play a role in stabilizing the molten globule (Uchiyama *et al.*, 1995). It is important to note that stabilization by non-specific hydrophobic interactions and the existence of specific native-like packing are not mutually exclusive. Non-specific hydrophobic interactions may help establish the overall native-like fold of the molten globule, while specific core packing may help stabilize nascent structure. Thus, the existence of some specific packing in the core of the α-LA molten globule does not preclude the importance of non-specific hydrophobic interactions in stabilizing molten globule structure. In addition, formation of a unique native structure likely requires close complementary side-chain packing. It will be interesting to determine whether, in the course of protein folding, specific packing helps stabilize structure arising from non-specific hydrophobic interactions.

Materials and Methods

Protein production

Full length α-$LA_{28\text{-}111}$ and variants thereof were produced as described previously (Peng *et al.*, 1995b). In summary, mutations were introduced by single-stranded mutagenesis and verified by DNA sequencing. Inclusion bodies of expressed proteins were washed with sucrose and Triton buffers, solubilized and reduced in urea/DTT, and purified by anion exchange chromatography and reverse phase HPLC. Reduced proteins were oxidized in 4 M GdnHCl, 0.1 M Tris (pH 8.8), for 48 hours at room temperature and further purified by reverse phase HPLC. Protein identity was confirmed by mass spectrometry.

Sedimentation equilibrium

Sedimentation equilibrium experiments were performed on a Beckman XL-A analytical ultracentrifuge as described previously (Peng *et al.*, 1995b). Protein solutions were dialyzed overnight against 10 mM Tris, 0.5 mM EDTA (pH 8.8), loaded at an initial concentration of 20 μM, and analyzed at 23,000 and 27,000 rpm, 0°C. Data were acquired at three wavelengths per rotor speed and processed simultaneously using a non-linear least squares fitting routine (Nonlin: Johnson *et al.*, 1981). Solvent density and protein partial specific volume were calculated according to solvent and protein composition, respectively (Laue *et al.*, 1992). The data fit well to a model for an ideal monomer (±5%), with no systematic deviation of the residuals.

Circular dichroism (CD) spectroscopy

CD spectroscopy was performed with an Aviv 62 DS spectrometer as described previously (Peng *et al.*, 1995b). Proteins were dissolved in 10 mM Tris, 0.5 mM EDTA (pH 8.8), to a concentration of 10 μM, and spectra were acquired at 0°C. Protein concentrations were determined by absorbance at 280 nm in 6 M GuHCl, 20 mM sodium phosphate (pH 6.5), using an extinction coefficient calculated based on tryptophan, tyrosine, and disulfide content (Edelhoch, 1967).

Effective concentration measurements

Effective concentration measurements (C_{eff}) were performed in an anaerobic chamber (Coy Laboratory Products). Native buffer consists of 10 mM Tris, 0.5 mM EDTA (pH 8.8). Denaturing buffer consists of 6 M GdnHCl, 10 mM Tris, 0.5 mM EDTA (pH 8.8). All buffers were degassed and stored under anaerobic conditions. Solutions of oxidized glutathoine (GSSG), reduced glutathoine (GSH), and protein were prepared fresh in buffer. The concentration of GSSG was determined spectroscopically at 248 nm using an extinction coefficient of 382 M^{-1} cm^{-1} (Chau & Nelson, 1991). 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). Samples of 5 μM protein in native or denaturing buffer were equilibrated for 28, 72, and 96 hours (native buffer) or 12 and 24 hours (denaturing buffer), starting from both oxidized and reduced proteins, at room temperature (23 to 27°C). Native and denaturing buffers contained GSH and GSSG in concentrations that established a glutathione reference redox of 1 M and 0.5 M (native buffer) or 5 mM (denaturing buffer), where the reference redox is defined as $[GSH]^2/[GSSG]$. Reactions were quenched by addition of 20% (v/v) acetic acid and analyzed by reverse phase HPLC. The amounts of oxidized and reduced proteins were determined by integrating peak areas.

Acknowledgements

We thank Zheng-yu Peng, Brenda Schulman, Debra Ehrgott, Michael Root, Dan Minor, and members of the Kim laboratory for helpful discussions and comments on the manuscript. This research was supported by the Howard Hughes Medical Institute.

References

Acharya, K. R., Ren, J., Stuart, D. I., Phillips, D. C. & Fenna, R. E. (1991). Crystal structure of human α-lactalbumin at 1.7 Å resolution. *J. Mol. Biol.* **221**, 571–581.

Alexandrescu, A. T., Evans, P. A., Pitkeathly, M., Baum, J. & Dobson, C. M. (1993). Structure and dynamics of the acid-denatured molten globule state of alpha-lactalbumin: a two-dimensional NMR study. *Biochemistry*, **32**, 1707–1718.

Bowie, J. U., Reidhaar-Olson, J. R., Lim, W. A. & Sauer, R. T. (1990). Deciphering the message in protein sequences: tolerance to amino acid substitutions. *Science*, **247**, 1306–1310.

Carra, J. H., Anderson, E. A. & Privalov, P. L. (1994). Thermodynamics of staphylococcal nuclease denaturation. II. The A-state. *Protein Sci.* **3**, 952–959.

Chau, M.-H. & Nelson, J. W. (1991). Direct measurement of the equilibrium between glutathione and dithiothreitol by high performance liquid chromatography. *FEBS Letters,* **291**, 296–298.

Colon, W. & Roder, H. (1996). Kinetic intermediates in the formation of the cytochrome *c* molten globule. *Nature Struct. Biol.* **3**, 1019–1025.

Colon, W., Elove, G. A., Wakem, L. P., Sherman, F. & Roder, H. (1996). Side-chain packing of the N- and C-terminal helices plays a critical role in the kinetics of cytochrome *c* folding. *Biochemistry,* **35**, 5538–5549.

Creighton, T. E. (1983). An empirical approach to protein conformation stability and flexibility. *Biopolymers,* **22**, 49–58.

Dobson, C. M. (1992). Unfolded proteins, compact states, and molten globules. *Curr. Opin. Struct. Biol.* **2**, 6–12.

Edelhoch, H. (1967). Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry,* **6**, 1948–1954.

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70–77.

Feng, Y., Sligar, S. G. & Wand, A. J. (1994). Solution structure of apocytochrome B562. *Nature Struct. Biol.* **1**, 30–35.

Hooke, S. D., Radford, S. E. & Dobson, C. M. (1994). The refolding of human lysozyme: a comparison with the structurally homologous hen lysozyme. *Biochemistry,* **33**, 5867–5876.

Johnson, M. L., Correia, J. J., Yphantis, D. A. & Halvorson, H. R. (1981). Analysis of data from the analytical ultracentrifuge by nonlinear least-squares techniques. *Biophys J.* **36**, 575–588.

Kay, M. S. & Baldwin, R. L. (1996). Packing interactions in the apomyoglobin folding intermediate. *Nature Struct. Biol.* **3**, 439–445.

Kuwajima, K. (1989). The molten globule state in protein folding. *Proteins: Struct. Funct. Genet,* **6**, 87–103.

Kuwajima, K. (1996). The molten globule state of α-lactalbumin. *FASEB J.* **10**, 102–109.

Laue, T. M., Shah, B. D., Ridgeway, T. M. & Pelletier, S. L. (1992). Computer-aided interpretation of analytical sedimentation data for proteins. In *Analytical Ultracentrifugation in Biochemistry and Polymer Science*, pp. 90–125, The Royal Society of Chemistry, Cambridge.

Lim, W. A. & Sauer, R. T. (1989). Alternative packing arrangements in the dydrophobic core of lambda repressor. *Nature,* **339**, 31–36.

Lin, L., Pinker, R. J., Forde, K., Rose, G. D. & Kallenbach, N. R. (1994). Molten globular characteristics of the native state of apomyoglobin. *Nature Struct. Biol.* **1**, 447–452.

Lin, T.-Y. & Kim, P. S. (1989). Urea dependence of thiol-disulfide equilibria in thioredoxin: confirmation of the linkage relationship and a sensitive assay for structure. *Biochemistry,* **28**, 5282–5287.

Lin, T.-Y. & Kim, P. S. (1991). Evaluating the effects of a single amino acid substitution on both the native and denatured states of a protein. *Proc. Natl Acad. Sci. USA,* **88**, 10573–10577.

Lumb, K. J. & Kim, P. S. (1995). A buried polar interaction imparts structural uniqueness in a designed heterodimeric coiled coil. *Biochemistry,* **34**, 8642–8648.

Marmorino, J. L. & Pielak, G. J. (1995). A native tertiary interaction stabilizes the A state of cytochrome *c*. *Biochemistry,* **34**, 3140–3143.

Matthews, B. W. (1995). Studies on protein stability with T4 lysozyme. *Advan. Protein Chem.* **46**, 249–278.

Morozova, L. A., Haynie, D. T., Arico-Muendel, C., Van Deal, H. & Dobson, C. M. (1995). Structural basis of the stability of a lysozyme molten globule. *Nature Struct. Biol.* **2**, 871–875.

Page, M. I. & Jencks, W. P. (1971). Entropic contributions to rate accelerations in enzymic and intramolecular reactions and the chelate effect. *Proc. Natl Acad. Sci. USA,* **68**, 1678–1683.

Peng, Z.-y. & Kim, P. S. (1994). A protein dissection study of a molten globule. *Biochemistry,* **33**, 2136–2141.

Peng, Z.-y. , Wu, L. C., Schulman, B. A. & Kim, P. S. (1995a). Does the molten globule have a native-like tertiary fold? *Phil. Trans. Roy. Soc. ser. B,* **348**, 43–47.

Peng, Z.-y. , Wu, L. C. & Kim, P. S. (1995b). Local structural preferences in the α-lactalbumin molten globule. *Biochemistry,* **34**, 3248–3252.

Pfeil, W., Bychkova, V. E. & Ptitsyn, O. B. (1986). Physical nature of the phase transition in globular proteins: calorimetric study of human α-lactalbumin. *FEBS Letters,* **198**, 287–291.

Ptitsyn, O. B. (1992). The molten globule state. In *Protein Folding* (Creighton, T. E., ed.), pp. 243–300, W. H. Freeman and Co., New York.

Redfield, C., Smith, R. A. & Dobson, C. M. (1994). Structural characterization of a highly-ordered ''molten globule'' at low pH. *Nature Struct. Biol.* **1**, 23–29.

Schulman, B. A. & Kim, P. S. (1996). Proline scanning mutagenesis of a molten globule reveals non-cooperative formation of a protein's overall topology. *Nature Struct. Biol.* **3**, 682–687.

Schulman, B. A., Redfield, C., Peng, Z.-y., Dobson, C. M. & 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.

Schulman, B. A., Kim, P. S., Dobson, C. M. & Redfield, C. (1997). A residue-specific NMR view of the non-cooperative unfolding of a molten globule. *Nature Struct. Biol.* **4**, 630–634.

Shimizu, A., Ikeguchi, M. & Sugai, S. (1993). Unfolding of the molten globule state of α-lactalbumin studied by ^{1}H NMR. *Biochemistry,* **32**, 13198–13203.

Song, J., Bai, P., Luo, L. & Peng, Z.-y. (1998). Contribution of individual residues to formation of the native-like tertiary topology in the α-lactalbumin molten globule. *J. Mol. Biol.* **280**, 167–174.

Uchiyama, H., Perez-Prat, E. M., Watanabe, K., Kumagai, I. & Kuwajima, K. (1995). Effects of amino acid substitutions in the hydrophobic core of α-lactalbumin on the stability of the molten globule state. *Protein Eng.* **8**, 1153–1161.

Wu, L. C. & Kim, P. S. (1997). Hydrophobic sequence minimization of the α-lactalbumin molten globule. *Proc. Natl Acad. Sci. USA,* **94**, 14314–14319.

Wu, L. C., Peng, Z.-y. & Kim, P. S. (1995). Bipartite structure of the α-lactalbumin molten globule. *Nature Struct. Biol.* **2**, 281–286.

Xie, D., Bhakuni, V. & Freire, E. (1991). Calorimetric determination of the energetics of the molten globule intermediate in protein folding: apo-α-lactalbumin. *Biochemistry,* **30**, 10673–10678.

Yutani, K., Ogasahara, K. & Kuwajima, K. (1992). Absence of the thermal transition in apo-α-lactalbumin in the molten globule state. *J. Mol. Biol.* **228**, 347–350.

Edited by P. E. Wright

(Received 8 January 1998; received in revised form 30 March 1998; accepted 31 March 1998)