Proline Scanning Mutagenesis Reveals Non-Native Fold in the Molten Globule State of Equine β-Lactoglobulin†

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ABSTRACT: The secondary structure in the molten globule state (an equilibrium analogue of a burst-phase folding intermediate) of equine β-lactoglobulin was investigated by changes in the circular dichroic spectrum induced by a series of site-directed proline substitutions. The results challenge the structural picture obtained from previous hydrogen/deuterium exchange experiments. A stable non-native α-helix was found to exist in the region corresponding to the eighth strand (H strand) in the native structure, where the backbone amide protons are the most strongly protected from exchange. Therefore, the backbone topology in the folding core is significantly different from that in the native structure. This indicates that the burst-phase folding intermediate of β-lactoglobulin is a trapped species because of misfolded backbone topology.

The mechanism through which a polypeptide chain folds into a unique 3D structure is not fully understood. Many theoretical and experimental studies on small globular proteins have shown that protein folding is a stochastic process. It is also known, however, that larger proteins (molecular mass of more than 10,000 Da) generally populate one or more specific folding intermediates (*1*, *2*). The molten globule is a commonly observed intermediate that shares compact conformation and significant secondary structures with the native state. Detailed structural characterization has been achieved for several model proteins, including apomyoglobin, cytochrome *c*, ribonuclease H, and α-lactalbumin (*3*, *4*). The results from these studies indicate that the molten globule contains a subdomain in which helices interact with each other in a native-like manner. However, the status of β-sheet proteins has not yet been clarified in equivalent detail.

β-Lactoglobulin is a major whey protein of 162 residues and is a member of lipocalin family, which shares an 8-stranded up-and-down β-barrel fold with a major α-helix located along the barrel (*5*). It is of particular interest that CD stopped-flow experiments suggest non-native α-helix formation during an early stage of the folding of this protein (*6*−*9*). To identify the non-native α-helix, Kuwata et al. (*10*) carried out NMR-detected pulse-labeling experiments on bovine β-lactoglobulin (BLG[1]). The results revealed that the amide protons in the regions corresponding to strands F, G, and H and a major *C*-terminal helix are well protected within 2 ms of refolding. Weak protection was also observed for residues 12−21, which correspond to a 3_{10}-helix and an *N*-terminal half of the A strand. They concluded that strands F, G, and H and the major *C*-terminal helix form a native-like core domain and that a non-native α-helix is formed in the *N*-terminal region.

A similar result was obtained for equine β-lactoglobulin (ELG), for which the protection from hydrogen/deuterium (H/D) exchange of backbone amides was measured for an equilibrium analogue of a folding intermediate (*11*). The equilibrium intermediate, the A state, is stable at acidic pH (*11*, *12*) and has been shown by CD spectra to be indistinguishable from a burst-phase folding intermediate (*9*). The backbone amide protons of ELG in the regions corresponding to strands A′ (*C*-terminal half of A), F, G, and H and a major *C*-terminal helix are protected from H/D exchange in the A state (Figure 1). Although the amino acid sequence of ELG differs significantly from that of BLG (sequence identity is only 57%), the partially folded subdomain is generally observed for β-lactoglobulins. These studies suggest that the molten globule contains a native-like subdomain for both β-sheet and α-helical proteins.

Protection from H/D exchange itself does not tell us which kind of secondary structure exists in these regions. Interpretations of previous studies rely on the native secondary structure of the protein. To analyze the secondary structure in the A state, a proline scanning mutagenesis experiment was performed. This method is based on the idea that the CD spectrum will change if a proline was substituted for a residue involved in an element of secondary structure and has been successfully used to identify the helical regions in the molten globule state of α-lactalbumin (*13*). It may be possible to distinguish between an α-helix and a β-sheet because proline is known to destabilize both α-helix and β-sheet structures and because these two types of structures show different CD spectral changes when they are disrupted.

Wild-type ELG has two disulfide bonds, Cys66−Cys160 and Cys106−Cys119 (Figure 1). Previously, recombinant ELG was expressed in *E. coli* and obtained in an insoluble fraction of cell lysate (*11*). It could be solubilized in concentrated urea and refolded by dilution with the buffer containing disulfide-shuffling reagents. It was expected that

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[1] Abbreviations: BLG, bovine β-lactoglobulin; ELG, equine β-lactoglobulin; CD, circular dichroism; GdnHCl, guanidine hydrochloride.

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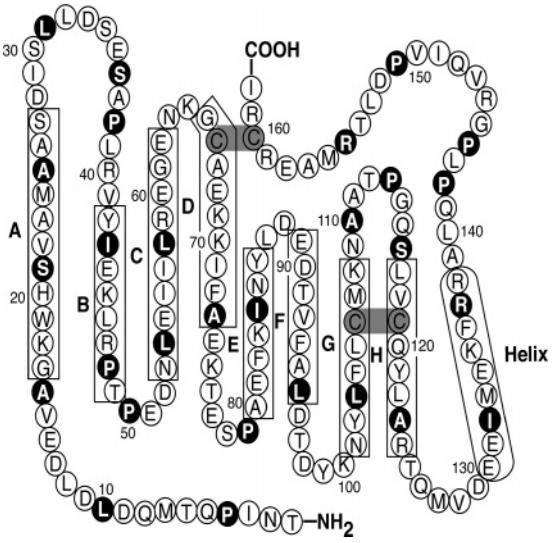

FIGURE 1: Schematic diagram showing the amino acid sequence and the secondary structure of ELG. The proline-substituted residues and endogenous prolines are shown in black. Cystine residues are shown in gray. The location of strands A−H and the major α-helix are based on the crystal structure of BLG (*28*).

proline-substituted mutants could not acquire a native-like tertiary structure and that non-native pairs of cysteine residues formed disulfide bonds. To prevent the formation of disulfide isomers, we constructed a single disulfide mutant in which Cys66 and Cys160 were substituted with alanine (C66A/C160A) and used as a pseudo-wild-type (WT*) ELG for proline substitution. It has been shown that the WT* produces CD spectra similar to those of the WT under conditions where the native and molten globule states are stabilized (*14*). Non-aromatic residues at various sites of the WT* sequence were substituted with proline, and the CD spectra of the WT* and the mutant in 0.1 M HCl−KCl (pH 1.5) at 25 °C were compared, where the A state is stable.

Recently, it has been found that the A state is converted to an expanded conformation with higher α-helical content at low temperature (*15*). In the case of C66A/C160A, a similar conformation was observed at low anion concentration and acidic pH (*14*). We refer to this conformational state as the C state. Yamada et al. (*15*) suggested that the secondary structures in the A state are stabilized by long-range hydrophobic interactions as well as local interactions, whereas the helices in the C state are stabilized predominantly by local interactions. Knowledge of the detailed structure of the C state is useful for understanding the stabilization mechanism of secondary structures in the partially folded species. Thus, the same set of proline-substituted mutants was used to study the structure of the C state.

MATERIALS AND METHODS

Materials. PCR primers were obtained from QIAGEN K.K. (Tokyo, Japan) and Operon Biotechnologies K. K. (Tokyo, Japan). BigDye Terminator v3.1 Cycle Sequencing kit was purchased from Applied Biosystems Japan (Tokyo, Japan). Other enzymes, chemicals, and kits for the molecular biological experiments were obtained from the suppliers as previously described (*11*). All other chemicals were of analytical grade from Nacalai Tesque (Kyoto, Japan) or Wako Pure Chemical Industries (Osaka, Japan).

Construction of Mutant ELG Genes and Expression Vectors. The ELG proline mutants L10P, A16P, L31P, L54P, and R137P were constructed using cassette mutagenesis as described previously (*11*). The other mutant genes were constructed by overlap extension (*16*). The DNA construct containing the disulfide deletion mutant (C66A/C160A) (*14*) was used as template DNA. The sequences of all mutants were confirmed by DNA sequencing on a Hitachi SQ-5500 or an ABI PRISM 3100-Avant. After sequence confirmation, the gene was inserted into the expression vector pET 3c that was transformed into *E. coli* BL21(DE3).

Expression, Refolding, and Purification of the Mutants. The mutant proteins were expressed, refolded, and purified as described for C66A/C160A (*14*). The purity of each sample was checked by SDS−PAGE and reverse-phase HPLC. It is especially important to analyze the sample by RP-HPLC because samples sometimes contain substances that are not stained with Coomassie brilliant blue but have UV absorbance (probably nucleic acids). Such impurities cause a serious problem because they result in an overestimate of the protein concentration and, hence, lower mean residue ellipticity. If such impurities were detected by RP-HPLC, they were separated from the protein by gel-filtration chromatography.

Circular Dichroism (CD) Measurements. CD spectra were measured with a Jasco J-720 spectropolarimeter. The protein concentration was 10−20 μM, as determined by UV spectroscopic measurements. Extinction coefficients of all mutants were assumed to be the same as that of C66A/C160A (12,000 M^{-1} cm^{-1}). Cuvettes with 1-mm and 10-mm path lengths were used for the measurements in the far- and near-UV regions, respectively. The standard error in ellipticity at 222 nm is less than 300 deg·cm^2/dmol, which was evaluated by measurements more than three times.

RESULTS AND DISCUSSION

Secondary Structures in the A State. Figure 2a shows the CD spectra of the WT* and several proline-substituted mutants in 0.1 M HCl−KCl (pH 1.5). The CD spectrum of the WT* has a shoulder at 222 nm and a minimum at 207 nm, which show that a significant amount of α-helix is present in the A state. The α-helix and β-sheet content is estimated to be 22% and 24%, respectively, as determined by a CONTIN/LL (*17*) analysis of the CD spectrum.

The double mutant I132P/R137P shows a reduced CD intensity at 222 nm and a minimum at a shorter wavelength compared with that of the WT*. This spectral change is typical of the unfolding of an α-helix. Both I132 and R137 are located in a major α-helix in the native structure, and the CD spectral change of I132P/R137P suggests that I132 and R137 assume a native-like α-helix in the A state. A similar spectral change was observed for A123P, although A123 is located in the H strand in the native conformation. A simple interpretation is that a region including A123 assumes a non-native α-helix in the A state. An alternative interpretation is that A123 and/or its neighboring residues interact with the region that is distant in the sequence and stabilize the helix. In such a case, A123P substitution would lead to a change in the interactions, resulting in the destabilization of the helix in that region. However, the latter case is unlikely. Figure 3a shows the CD intensities at 222

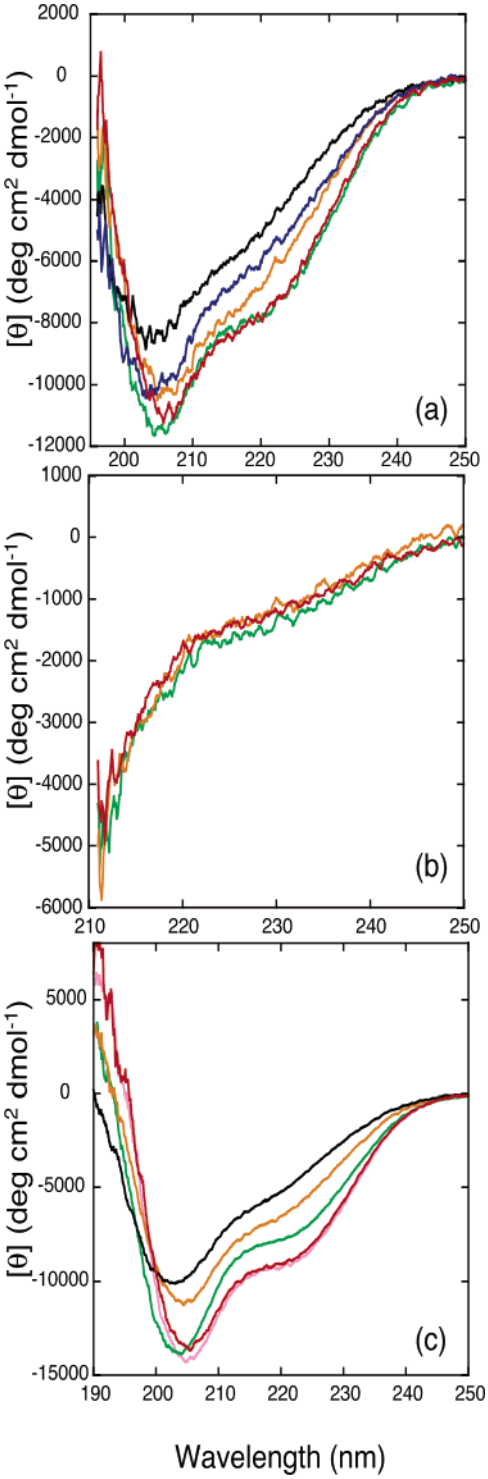

FIGURE 2: CD spectra of the WT* and several proline-substituted mutants in 0.1 M HCl−KCl at pH 1.5 (a), 6 M GdnHCl (b), and 0.1 M phosphoric acid at pH 1.7 (c). The spectra are color-coded as follows: WT* (red), L31P (pink), L103P (green), A123P (orange), A123P/I132P (black), and I132P/R137P (blue).

nm for all mutant proteins. Because all mutant proteins show similar CD spectra in the presence of 6 M GdnHCl (Figures 2b and 3), the spectral change observed in the A state can be ascribed to a structural change. Of the 19 mutated sites, the mutations that significantly affect CD intensity are limited to residues in the H strand (A123) and a major helix (I132 and R137). It should be noted that there are nine endogenous proline residues at positions 4, 38, 48, 50, 80, 113, 142, 144, and 151 (Figure 1). The mutated and endogenous sites are distributed throughout the ELG sequence, and the longest contiguous sequence that has not been probed by mutation is the segment from R59 to A72. However, this region contains two glycine residues at 61 and 65. Given that glycines are helix breakers (*18*), it is unlikely that the non-native helix exists in this region. That is, there is little possibility that the non-native α-helix is located in the regions not probed and that it is disrupted by A123P mutation. It is, therefore, concluded that a region including A123 assumes a non-native α-helix in the A state.

The substitution-sensitive sites, A123, I132, and R137, have been shown to be protected from H/D exchange, suggesting that these residues form hydrogen bonds in the A state (*11*). However, proline substitutions for some other protected residues do not cause significant CD spectral changes. L103 was shown to have a protection factor of 49 in the A state, suggesting that L103 forms a stable hydrogen bond. However, L103P shows almost the same CD intensity as that of the WT* (Figure 3a). In the previous H/D exchange study, the G and H strands were thought to form a native-like *β*-hairpin in the A state because these strands are linked by a disulfide bridge between Cys106 and Cys119, and they are the most strongly protected regions (*11*). If the GH *β*-hairpin is formed in the A state, it is expected that L103P and A123P would show similar CD spectral changes. The variation in CD spectral change for L103P and A123P is clear in the difference CD spectrum between mutants and the WT* (Figure 4) and suggests that L103 and A123 assume different kinds of ordered structure that protect backbone amide protons from exchange. The difference spectrum of A123P has negative peaks at 222 and 207 nm, indicating an α-helical conformation for A123. In contrast, the difference spectrum of L103P has a positive peak at 230 nm and a negative peak at 215 nm. It is interesting that the spectral change of L95P is similar to that of L103P. This shape of the difference spectrum for L95P and L103P is similar to a difference spectrum between a *β*-sheet and a random coil derived from protein CD spectra (*19*). These results, together with the fact that L95 has a protection factor of 12, suggest that the F and G strands may form a native-like *β*-hairpin. However, it is difficult to determine whether the hydrogen-bonded pairs between the F and G strands are native-like. There are many possible conformations because residues in the G strand region are contiguously protected (*11*). For example, the amide of L103 may form a hydrogen bond with the residues in the F strand, although it forms a hydrogen bond with the H strand in the native state. In either case, the amide protons that are in the G strand but not included in the hydrogen bond with the residues in the F strand would be included in hydrogen bonds with another region because the H-strand region assumes an α-helix.

In the previous study (*11*), weak protection against H/D exchange was observed for A23 and A25, and thus, the A and H strands were assumed to have a native-like *β*-sheet. However, our present results show that the H-strand region assumes a non-native α-helix. Therefore, it is unlikely that the A strand forms hydrogen bonds to the H strand. S21P and A25P do not show significant CD spectrum changes (Figure 3a), which excludes the possibility that a non-native α-helix is formed in this region. The difference spectra of S21P and A25P also differ from those of L95P and L103P

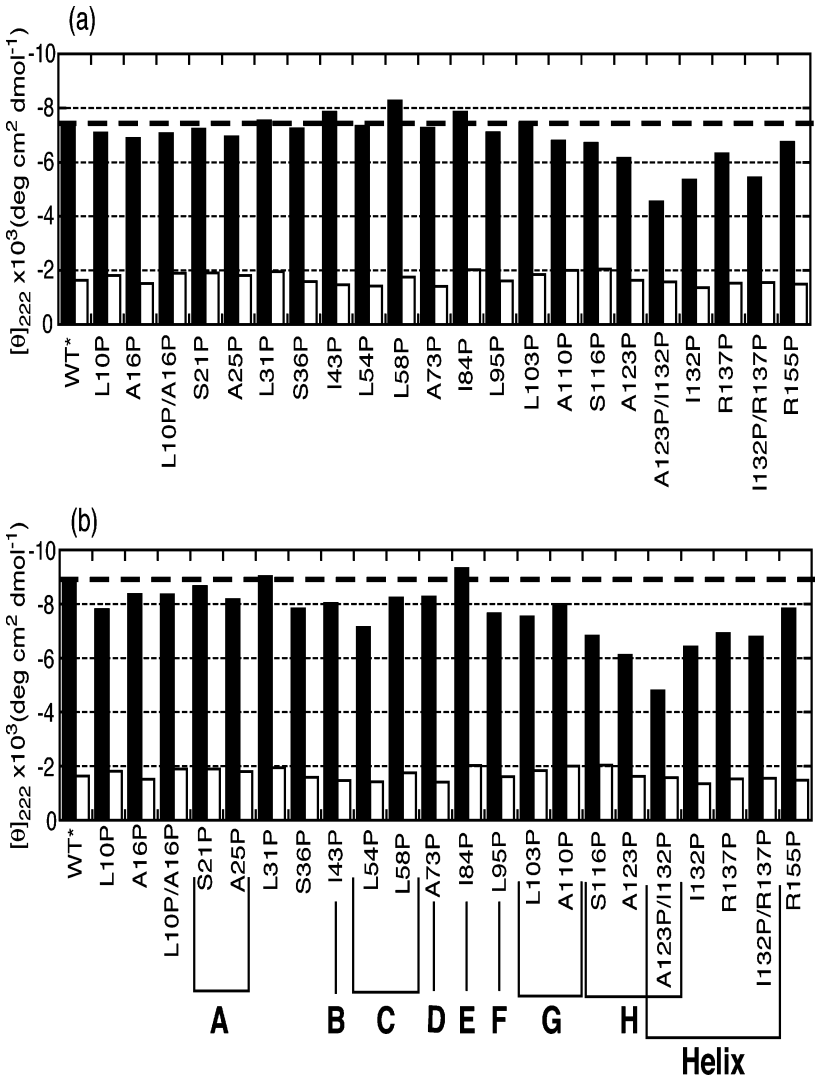

FIGURE 3: (a) $[\theta]_{222}$ for the WT* and all proline-substituted mutants in the A state (solid bars) and in 6 M GdnHCl (open bars). (b) $[\theta]_{222}$ for the WT* and all proline-substituted mutants in the C state (solid bars) and in 6 M GdnHCl (open bars). The dashed line represents $[\theta]_{222}$ for the WT*. The secondary structure elements to which the proline-substituted residues are included are shown below panel b.

(Figure 4). The amide protons of A23 and A25 might be protected by being buried in the interior of a compact globular structure in the A state.

Secondary Structures in the C State. Figure 2c shows the CD spectra of the WT* and several proline-substituted mutants in 0.1 M phosphoric acid (pH 1.7), where the WT* is known to be a helical and expanded conformational state (*14*). The CD intensity in this state is much stronger than that in the A state. A CONTIN/LL analysis indicated that the α-helix and β-sheet contents are 27% and 18%, respectively. Therefore, α-helix content increases and β-sheet content decreases when the molecule is expanded by electrostatic repulsion.

The pattern of CD intensity change (Figure 3b) is similar to that in the A state (Figure 3a). That is, the structured region in the C state is not significantly different from that in the A state. Remarkable differences are found in L95P and L103P. In contrast to the fact that their CD intensities at 222 nm are similar to the value of the WT* in the A state, they are weaker in the C state. Difference CD spectra (Figure 4) clearly show the structural difference in these regions between the A and C states. The difference spectrum in the A state suggests that the structure lost by proline substitution is not an α-helix. In the C state, however, the difference spectra of L95P and L103P are similar to that of I132P, indicating that the structure lost by proline substitution is an α-helix. In the C state, therefore, the regions corresponding to the F and G strands also assume non-native α-helices. L54P also shows a reduced CD intensity at 222 nm (Figure 3b), suggesting a non-native α-helix in this site. Furthermore, many other mutants show slightly reduced CD intensity, which may indicate that there are many unstable helices throughout the chain in the C state.

Effect of Double Mutations. To investigate whether proline substitution completely disrupts the secondary structure and whether the structures in the different regions are stabilized independently, several double mutants were prepared. In Figure 4, difference CD spectra are compared between A and C states. The CD spectrum changes of I132P and R137P indicate that the α-helix in this region is disrupted by proline substitution. In the C state, the difference in ellipticity at 222 nm of I132P/R137P is similar to those of the single-site mutants I132P and R137P, indicating that a single proline substitution is sufficient to completely disrupt this helix in the C state (Figures 3b and 4). The ellipticity change of I132P/R137P (about 1900 deg·cm^2·dmol^{-1}) corresponds to

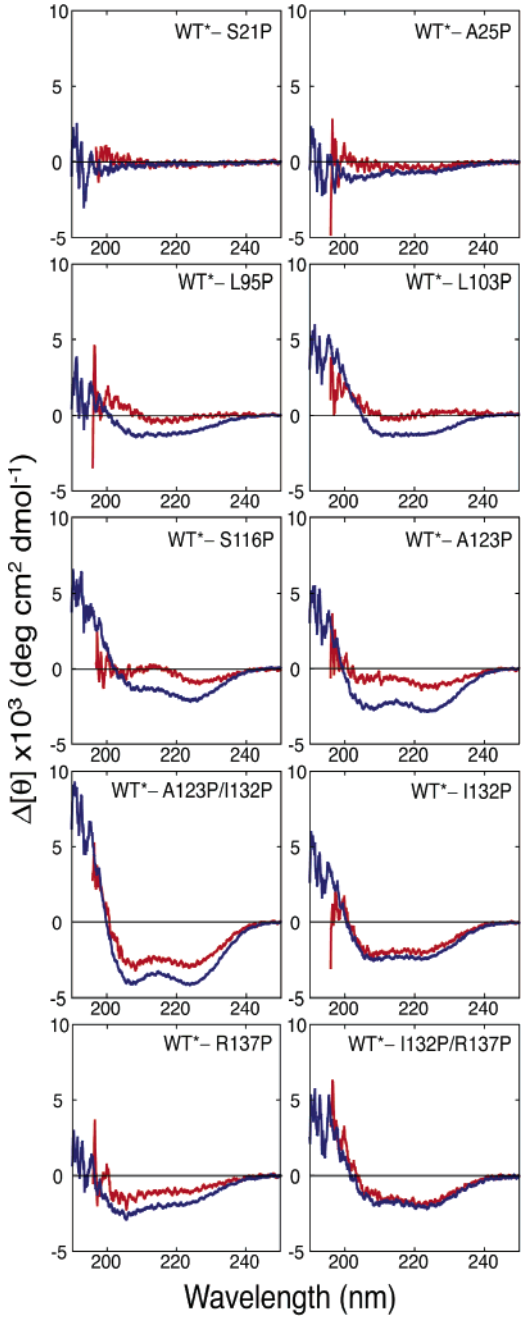

FIGURE 4: Difference CD spectra between the mutants and the WT*. These were calculated by subtracting the mutant spectrum from the WT* spectrum in 0.1 M HCl−KCl at pH 1.5 (A state; red) and 0.1 M phosphoric acid at pH 1.7 (C state; blue).

the unfolding of a helix of 7−9 residues in length because ellipticity at 222 nm is −37,000 to −40,000 when the entire polypeptide assumes a helical conformation (*19*, *20*). From the fact that I132P has reduced helix content, at least four preceding residues are in a helical conformation. A probable helix location is, therefore, from residues 128 to 137, being slightly shifted to the *N*-terminal side of the *C*-terminal helix in the native conformation. This is consistent with the result of the H/D exchange experiment. The residues in the *N*-terminal side of this helix are strongly protected in the A state, whereas those on the *C*-terminal side show stronger protection in the native state (*11*).

Because the difference spectrum of I132P/R137P in the A state is similar to that in the C state (Figure 4), the helix length in the A state is not different from that in the C state. The difference spectra of I132P, and especially R137P, in the A state are less intense than those in the C state, suggesting that a single proline substitution is not sufficient to disrupt the helix in the A state. Therefore, the *C*-terminal helix in the A state is more stable than that in the C state. It has been suggested that the secondary structures in the A state are stabilized by long-range hydrophobic interactions (*15*).

The difference spectrum of A123P is also typical of α-helix disruption both in the A and C states. The difference is larger in the C state than that in the A state, suggesting that the helix is longer in the C state or that a single proline substitution is not sufficient to disrupt the helix, which is more stable in the A state. The latter is more likely because the change in ellipticity at 222 nm (about 1200 deg·cm^2·dmol^{-1}) corresponds to only one turn of the α-helix, yet amide protons from V118 to A123 are strongly protected. In the C state, where the helix is expected to be less stable, the change in ellipticity (about 2700 deg·cm^2·dmol^{-1}) corresponds to 11−12 residues. A possible location of this helix is, therefore, from T112 to R124 because S116P also shows the spectrum change typical of helix disruption in the C state. Because the ellipticity change of A123P/I132P is nearly the same as the sum of ellipticity changes of A123P and I132P, the non-native helix located in the H strand region seems to be independent of the native-like *C*-terminal helix.

Kuwata et al. have reported weak protection against hydrogen exchange for residues 12−21 during the burst phase of BLG folding (*10*). In this study, no significant CD spectrum changes were observed for L10P, A16P, and S21P. We tested the effect of the double mutant, L10P/A16P, but it showed little CD spectral change. In the A and C states of ELG, therefore, the *N*-terminal region does not assume a non-native α-helix.

Implications for the Folding Mechanism. Recent studies have suggested non-native structure formation during folding reactions. Mok et al. (*21*) have shown that non-native side chain interactions in the molten globule state of α-lactalbumin stabilize a native-like fold. A sequence simplification experiment has also shown that nonspecific hydrophobic interactions may be sufficient to determine the fold in the molten globule (*22*). However, the existence of non-native side-chain interactions does not impose any significant barrier to the folding of this protein because the molten globule and unfolded states of bovine α-lactalbumin are kinetically equivalent (*23*). Nishimura et al. (*24*) found one-helical-turn translocation of the H helix in the burst-phase folding intermediate of apomyoglobin. This is similar to the situation of the *C*-terminal helix of ELG. However, mutation experiments on the residues at the helix interface have indicated that helix docking is topologically similar to that in the native structure.

The present study reveals that the molten globule structure, which was shown to be indistinguishable from the burst-phase folding intermediate, differs significantly from the native structure. This stands in contrast to the current model that the burst-phase intermediate assumes a native-like core consisting of the F, G, and H strands and the *C*-terminal helix. The results of this study clearly show that the H strand does not assume a native-like β-sheet structure but instead assumes a non-native α-helix. Therefore, the molten globule

of ELG probably assumes a non-native backbone topology in contrast to the molten globule states of apomyoglobin and α-lactalbumin. This suggests that the native-like backbone topology or subdomain formation is not a general property of the molten globule intermediate. Recently, it has been suggested that an *N*-terminal fragment of phosphoglycerate kinase, comprising an $(\alpha\beta)_6$ Rossman fold, assumes a helical bundle molten globule at low GdnHCl concentration, although this domain attains a native-like topology during the burst phase of folding (*25*).

In the case of β-lactoglobulin, the burst phase clearly produces a misfolded and trapped species rather than a productive folding intermediate. The non-native α-helix formed in the region corresponding to the H strand has considerable stability and must be unfolded before the protein can acquire the native conformation. This may be a rare case, because the protein sequence is generally designed to be consistent and not frustrated (*26*, *27*). However, it may be general that the molten globule observed during an early stage of folding is trapped because of its incorrect backbone fold, as in the case of β-lactoglobulin.

REFERENCES

1. Dobson, C. M. (2003) Protein folding and misfolding, *Nature 426*, 884−890.
2. Daggett, V., and Fersht, A. R. (2003) Is there a unifying mechanism for protein folding? *Trends Biochem. Sci. 28*, 18−25.
3. Baldwin, R. L., and Rose, G. D. (1999) Is protein folding hierarchic? II. Folding intermediates and transition states, *Trends Biochem. Sci. 24*, 77−83.
4. Arai, M., and Kuwajima, K. (2000) Role of the molten globule state in protein folding, *Adv. Protein Chem. 53*, 209−282.
5. Sawyer, L., and Kontopidis, G. (2000) The core lipocalin, bovine beta-lactoglobulin, *Biochim. Biophys. Acta 1482*, 136−148.
6. Kuwajima, K., Yamaya, H., Miwa, S., Sugai, S., and Nagamura, T. (1987) Rapid formation of secondary structure framework in protein folding studied by stopped-flow circular dichroism. *FEBS Lett. 221*, 115−118.
7. Kuwajima, K., Yamaya, H., and Sugai, S. (1996) The burst-phase intermediate in the refolding of beta-lactoglobulin studied by stopped-flow circular dichroism and absorption spectroscopy, *J. Mol. Biol. 264*, 806−822.
8. Hamada, D., Segawa, S., and Goto, Y. (1996) Non-native alpha-helical intermediate in the refolding of beta-lactoglobulin, a predominantly beta-sheet protein, *Nat. Struct. Biol. 3*, 868−873.
9. Fujiwara, K., Arai, M., Shimizu, A., Ikeguchi, M., Kuwajima, K., and Sugai, S. (1999) Folding-unfolding equilibrium and kinetics of equine beta-lactoglobulin: equivalence between the equilibrium molten globule state and a burst-phase folding intermediate, *Biochemistry 38*, 4455−4463.
10. Kuwata, K., Shastry, R., Cheng, H., Hoshino, M., Batt, C. A., Goto, Y., and Roder, H. (2001) Structural and kinetic characterization of early folding events in beta-lactoglobulin, *Nat. Struct. Biol. 8*, 151−155.
11. Kobayashi, T., Ikeguchi, M., and Sugai, S. (2000) Molten globule structure of equine beta-lactoglobulin probed by hydrogen exchange, *J. Mol. Biol. 299*, 757−770.
12. Ikeguchi, M., Kato, S., Shimizu, A., and Sugai, S. (1997) Molten globule state of equine beta-lactoglobulin, *Proteins 27*, 567−575.
13. Schulman, B. A., and Kim, P. S. (1996) Proline scanning mutagenesis of a molten globule reveals non-cooperative formation of a protein's overall topology, *Nat. Struct. Biol. 3*, 682−687.
14. Yamada, Y., Nakagawa, K., Yajima, T., Saito, K., Tokushima, A., Fujiwara, K., and Ikeguchi, M. (2006) Structural and thermodynamic consequences of removal of a conserved disulfide bond from equine beta-lactoglobulin, *Proteins 63*, 595−602.
15. Yamada, Y., Yajima, T., Fujiwara, K., Arai, M., Ito, K., Shimizu, A., Kihara, H., Kuwajima, K., Amemiya, Y., and Ikeguchi, M. (2005) Helical and expanded conformation of equine beta-lactoglobulin in the cold-denatured state, *J. Mol. Biol. 350*, 338−348.
16. Sambrook, J., and Russell, D. W. (2001) *Molecular Cloning : A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Plainview, NY.
17. Sreerama, N., and Woody, R. W. (2000) Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set, *Anal. Biochem. 287*, 252−260.
18. Bryson, J. W., Betz, S. F., Lu, H. S., Suich, D. J., Zhou, H. X., O'Neil, K. T., and DeGrado, W. F. (1995) Protein design: a hierarchic approach, *Science 270*, 935−941.
19. Chen, Y. H., Yang, J. T., and Chau, K. H. (1974) Determination of the helix and beta form of proteins in aqueous solution by circular dichroism, *Biochemistry 13*, 3350−3359.
20. Scholtz, J. M., Barrick, D., York, E. J., Stewart, J. M., and Baldwin, R. L. (1995) Urea unfolding of peptide helices as a model for interpreting protein unfolding, *Proc. Natl. Acad. Sci. U.S.A. 92*, 185−189.
21. Mok, K. H., Nagashima, T., Day, I. J., Hore, P. J., and Dobson, C. M. (2005) Multiple subsets of side-chain packing in partially folded states of alpha-lactalbumins, *Proc. Natl. Acad. Sci. U.S.A. 102*, 8899−8904.
22. Wu, L. C., and Kim, P. S. (1997) Hydrophobic sequence minimization of the alpha-lactalbumin molten globule, *Proc. Natl. Acad. Sci. U.S.A. 94*, 14314−14319.
23. Ikeguchi, M., Fujino, M., Kato, M., Kuwajima, K., and Sugai, S. (1998) Transition state in the folding of alpha-lactalbumin probed by the 6-120 disulfide bond, *Protein Sci. 7*, 1564−1574.
24. Nishimura, C., Dyson, H. J., and Wright, P. E. (2006) Identification of native and non-native structure in kinetic folding intermediates of apomyoglobin, *J. Mol. Biol. 355*, 139−156.
25. Reed, M. A., Jelinska, C., Syson, K., Cliff, M. J., Splevins, A., Alizadeh, T., Hounslow, A. M., Staniforth, R. A., Clarke, A. R., Craven, C. J., and Waltho, J. P. (2006) The denatured state under native conditions: a non-native-like collapsed state of N-PGK, *J. Mol. Biol. 357*, 365−372.
26. Go, N. (1983) Theoretical studies of protein folding, *Annu. Rev. Biophys. Bioeng. 12*, 183−210.
27. Bryngelson, J. D., Onuchic, J. N., Socci, N. D., and Wolynes, P. G. (1995) Funnels, pathways, and the energy landscape of protein folding: a synthesis, *Proteins 21*, 167−195.
28. Brownlow, S., Morais Cabral, J. H., Cooper, R., Flower, D. R., Yewdall, S. J., Polikarpov, I., North, A. C., and Sawyer, L. (1997) Bovine beta-lactoglobulin at 1.8 Å resolution—still an enigmatic lipocalin, *Structure 5*, 481−495.

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