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A Synthetic Peptide Study on the Molten Globule of α-Lactalbumin[1]

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We investigated the conformations of peptides that encompass the B helix or C helix region formed in the molten globule of bovine α-lactalbumin to get information on the molecular mechanism that stabilizes the molten globule. The CD spectra show that the isolated B and C helices are intrinsically unstable. The chemical shifts, NOE connectivities, and CD spectrum indicate that no helical structure is induced in the C helix region (86-99) by extending the peptide sequence to include the hydrophobic cluster region (101-107), although the hydrophobic cluster region can be regarded as a possible initiation site for folding of the protein. We also clarified that the isolated B helix (23-34) peptide does not directly interact with the C helix or hydrophobic cluster region. These results suggest that the B and C helices in the molten globule are stabilized by their interaction with other parts of the protein.

Key words: α-lactalbumin, helix, hydrophobic cluster, molten globule, peptide.

The molten globule state is a well-defined equilibrium intermediate state that shares many properties of a transiently populated intermediate in the course of folding. The characteristic features of the molten globule state are a substantial content of a secondary structure which is presumed to be largely native-like, a compactness which is similar to that of the native state, but substantially disordered tertiary interactions (*1-3*). The most extensively characterized molten globule state is that of α-lactalbumin which is obtained by acid-induced unfolding at pH 2 or by removing a bound Ca^{2+} at neutral pH (*1, 4*).

The native conformation of α-lactalbumin is composed of two domains, α-domain and β-domain. The former is formed by four α-helices, A (5-11), B (23-34), C (86-99), and D (105-109), and the latter contains a small antiparallel β-sheet (40-50) (*5-8*) (Fig. 1). It has recently been shown by disulfide rearrangement experiments with mutant α-lactalbumin that the α-domain has a native-like backbone topology and the β-domain is predominantly disordered in the molten globule state (*9, 10*). This is consistent with the result that limited proteolysis of the molten globule state occurs at sites located in the β-domain (*11*). Two-dimensional NMR studies utilizing hydrogen exchange and pH-jump have also shown that the B and C helices are persistent in the molten globule (*12-14*). Furthermore, a direct NMR investigation of the molten globule has indicated that a non-native hydrophobic cluster is formed in the 101-107 region, which is located between the B and C helices in the native structure (*13*) (Fig. 1). These results suggest that the B and C helices and the 101-107 region interact with each other and stabilize a core structure in the molten globule. However, the detailed mechanism of stabilization of the molten globule, *i.e.*, the interaction operative at an early stage of protein folding, is not yet fully understood. One way to dissect interactions responsible for stabilization of the molten globule structure is to investigate the effect of amino acid substitution on the stability of the structure (*15-17*); another is to ask whether a part of the molecule can assume a local structure observed in the whole molecule (*9, 18*). Uchiyama *et al.* (*19*) have recently shown that replacements of Thr or Ala residues in the B helix with Ile result in stabilization of the molten globule state of α-lactalbumin and suggested the importance of hydrophobic interaction in this stabilization.

In this study, we synthesized three peptides that encompass the helices or the hydrophobic cluster region formed in the molten globule of bovine α-lactalbumin to get further information on the molecular mechanism of stabilizing the molten globule. The peptides B14 and C17 were synthesized to investigate the intrinsic helical propensities of the B and C helices. These peptides correspond to the regions 22-35 and 84-100 and encompass the B and C helices, respectively. The peptide C24 (residue 84-107) was also synthesized to examine whether the C helix is stabilized by interaction with the hydrophobic cluster in the 101-107 region. Furthermore, we studied whether B14 and C24 can interact noncovalently with each other and stabilize the helices, even though much of the intervening polypeptide chain is absent.

MATERIALS AND METHODS

Materials—The sequences of peptides used in this study are shown in Table I. The peptides were synthesized on EXCELL or 9050 peptide synthesizers (MilliGen/Biosearch). Fluorenylmethyloxy-carbonyl (Fmoc) amino acids were coupled onto the PAL resin (MilliGen/Biosearch) so that the resultant peptides have an amide group at the C terminus. For coupling the Cys residue, *N*-Fmoc-*S*-acetamidomethylcysteine was used, leaving the *S*-acetamidomethyl group in the peptides. Before cleavage of the peptides from resins, portions of the peptides were *N*-acetylated with *N*-acetyl imidazol (AcB14, AcC17,

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Abbreviation: NOE, nuclear Overhauser enhancement.

TABLE I. **Peptide sequence of H7, B14, C17, and C24.**

Peptide	Sequence
*	84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107
	C-helix in the N-state
H7	L N Y W L A H (NH_2)
C17	D L T D D I M C V K K I L N K V G (NH_2)
C24	D L T D D I M C V K K I L N K V G L N Y W L A H (NH_2)
*	22 23 24 25 26 27 28 29 30 31 32 33 34 35
	B-helix in the N-state
B14	S L P E W V C T T F H T S G (NH_2)

*The number shows the amino acid position of bovine α-lactalbumin

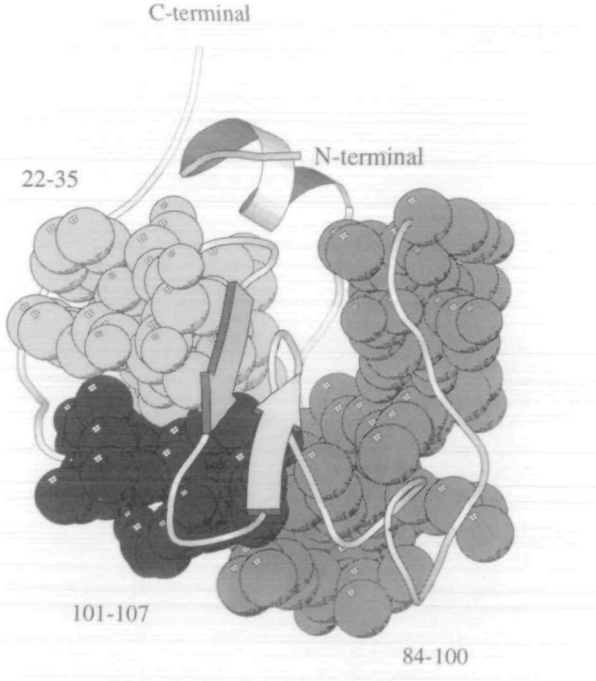

Fig. 1. Schematic drawing of the native structure of baboon α-lactalbumin (*5*) produced with the program MolScript (*43*). The polypeptide chain of the whole molecule is represented as a ribbon model viewed from the β-domain side. The regions that correspond to the peptides synthesized in this study are shown as CPK models.

AcC24). The peptides were purified by reverse-phase HPLC (Waters μBondasphere C18-100 Å 19×150 mm). The amino acid sequences of unacetylated peptides were verified by amino acid sequencing with an Applied Biosystems 473A protein sequencer. Molecular weights of the *N*-acetylated peptides were determined by FAB mass spectroscopy using a JEOL SX-102.

D_2O (99.96% D) was obtained from Isotec. DCl (99.5% D) was obtained from Aldrich. NaOD (99.5% D) and TSP were purchased from Merck.

CD Measurements—CD measurements were made at 5 or 35°C using a Jasco J-720 spectropolarimeter that was calibrated with *d*-10-camphorsulfonic acid. Samples were prepared in 1 mM citrate buffer of which ionic strength was adjusted to 0.01 with KCl (peptide concentration was 4-500 μM). Quartz cells with path lengths of 1 and 0.1 mm were used depending on the sample concentration. The concentration of a stock solution of AcC17 was determined by comparison of the peak area of ^{1}H NMR spectrum of AcC17 and that of the internal reference TSP. The AcB14 and AcC24 peptides concentrations were determined by assuming molar extinction coefficients at 280 nm for tryptophan and tyrosine residues of 5,559 and 1,197 $M^{-1}\cdot cm^{-1}$, respectively (*20*). The mean residue ellipticity, $[\theta]$, was calculated from the relationship $[\theta]=\theta\times 1/(10lcN)$, where θ is the observed ellipticity, l is the path length in cm, c is molar concentration of the peptide, and N is the number of amino acid residues in the sequence.

NMR Measurements—Samples of the peptides were prepared for NMR measurements in water (90% H_2O/10% D_2O). The pH was adjusted to 2.0 with DCl. All pH values were measured at room temperature, and not corrected for deuterium isotope effect. TSP was used as an internal chemical shift standard. NMR spectra were obtained on a JEOL A-500 spectrometer at 35°C. Sequence-specific assignments were achieved by DQF-COSY (*21*), HOHAHA (*22*), NOESY (*23*), and ROESY (*24*). All two-dimensional ^{1}H-NMR spectra were acquired in the phase-sensitive mode with the TPPI-States method (*25*). HOHAHA were recorded with spin-locking of longitudinal magnetization for periods of 60 or 90 ms. NOESY spectra were recorded with mixing times of 250-350 ms Water suppression was achieved by presaturation. A total of 512 FIDs of 32 scans was collected with 1,024 data points over a spectral width 8,000 Hz, with a 1.5 s recycle delay. Spectra were resolution enhanced by shifted sine-bell functions in both dimensions.

RESULTS

CD spectra of AcB14 and AcC17 at pH 2 and 5°C are shown in Fig. 2. For these peptides, the diagnostic helical signals at 208 and 222 nm are not observed, and a trough around at 200 nm is observed. This feature of the CD spectrum is typical of random coil conformation (*26*). The CD spectra of these peptides were essentially identical at any pH from 2 to 12, and addition of 0.1 M KCl did not induce any CD spectrum change. No indication of an ordered structure was obtained by ^{1}H NMR (see below). These results indicate that the B and C helices of α-lactalbumin are intrinsically unstable and that they are stabilized in the molten globule state by their interaction with other parts of the protein. The CD spectrum of AcC24 also indicates that AcC24 does not adopt significant helical structures (Fig. 2). To confirm that no helical structure at all is induced in AcC24 compared with AcC17, however, it is necessary to know the contribution of the region 101-107 to the CD spectrum of AcC24. Therefore, we synthesized an additional peptide corresponding to the region 101-107 (H7). To make the

total number of peptide bonds of H7 and AcC17 equal to the number of peptides in AcC24, the N-terminus of H7 was not acetylated. The CD spectrum of H7 is shown in Fig. 3. The positive CD band at 225 nm probably results from aromatic CD contributions (*27*). In Fig. 3, the calculated CD spectrum by adding the spectra of H7 and AcC17 is compared with the spectrum of AcC24. Although the CD spectrum of AcC24 differs from the sum of the spectra of H7 and AcC17, it is not clear whether the difference corresponds to the helix formation. We therefore investigated the conformations of C17 and C24 by ^{1}H NMR. Because AcC24 aggregates at higher concentration (>500 μM), we used C17 and C24 instead of their acetylated counterparts in the NMR study. The CD spectra of C17 and C24 are identical with those of AcC17 and AcC24, respectively. The complete assignments of proton resonances of the peptides were achieved using the sequential assignment procedure (*28*). The sequential NOE between amide protons and the medium-range NOE have not been observed for C17. Figure 4 shows the chemical shift deviations from the random coil values (*28*) for all observable $C_\alpha H$. It is well known that the chemical shift of $C_\alpha H$ is sensitive to the backbone conformation of polypeptide chain (*29, 30*). The chemical shift deviations of C17 are less than 0.1 ppm, except for the N-terminal residues. These NMR results as well as the CD spectrum indicate that C17 does not assume any ordered structure. Figure 4 also shows the chemical shift deviations of C24. The chemical shifts of the region 84-100 in C24 coincide well with those of C17, indicating that no helical structure is induced in the C helix region by extending the peptide sequence to include the hydrophobic cluster region. The C-terminal region of C24 (101-107) shows significant deviation of the $C_\alpha H$ chemical shift from the random coil value. Similar shifts were observed for H7 peptide (Fig. 4). It is not clear whether these significant deviations from the random coil values result from the detectable levels of structured conformations or from the ring current effect of Y103 or W104. However, observation of several NOE cross-peaks between NH resonances in the region 101-105 suggests that a nonrandom conformation is formed in the region 101-105 (*Fig. 5*). Although no medium-range NOE was observed and we have not been able to define the structure formed in the region 101-105, this result suggests that the region 101-105 may be an initiation site for protein folding.

If the B and C helices in the molten globule state are stabilized by interaction with each other or with the hydrophobic cluster in the region 101-107, the helix may be induced by complex formation of AcB14 and C24. We therefore examined whether the CD spectrum is changed by mixing AcB14 and C24. In this mixing experiment, the

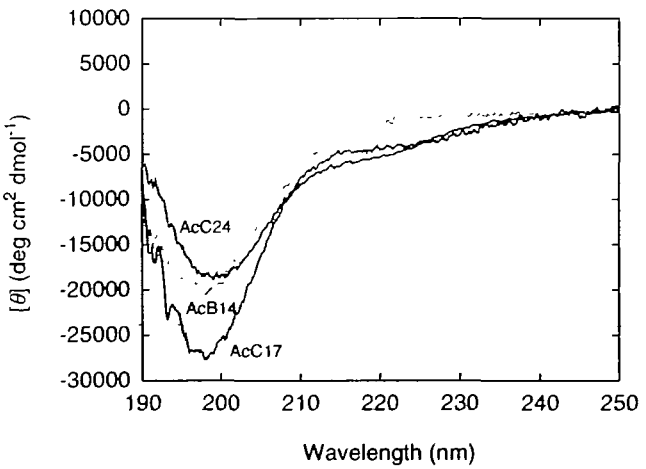

Fig. 2. **CD spectra of AcB14, AcC17, and AcC24 at pH 2.0 and 5°C.**

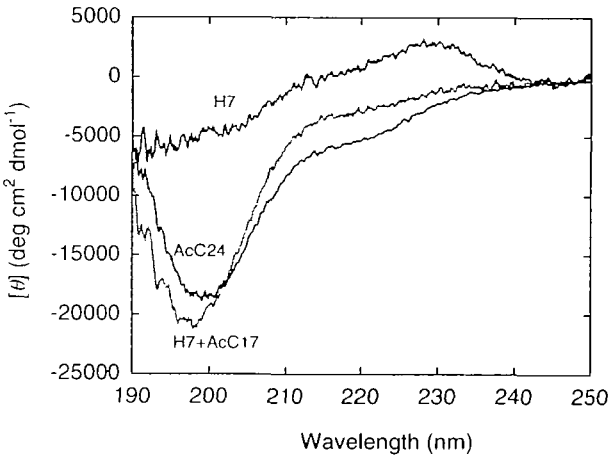

Fig. 3. **Comparison of the CD spectrum of AcC24 with the sum of the spectra of AcC17 and H7.** The CD spectrum of H7 is also shown. Conditions are the same as in Fig. 2. The spectrum summation was done with the following equation: $[\theta]_\lambda^{sum}=(17\times[\theta]_\lambda^{AcC17}+7\times[\theta]_\lambda^{H7})/24$, where $[\theta]_\lambda^{AcC17}$ and $[\theta]_\lambda^{H7}$ are the mean residue ellipticities of AcC17 and H7 peptide, respectively, at the wavelength λ.

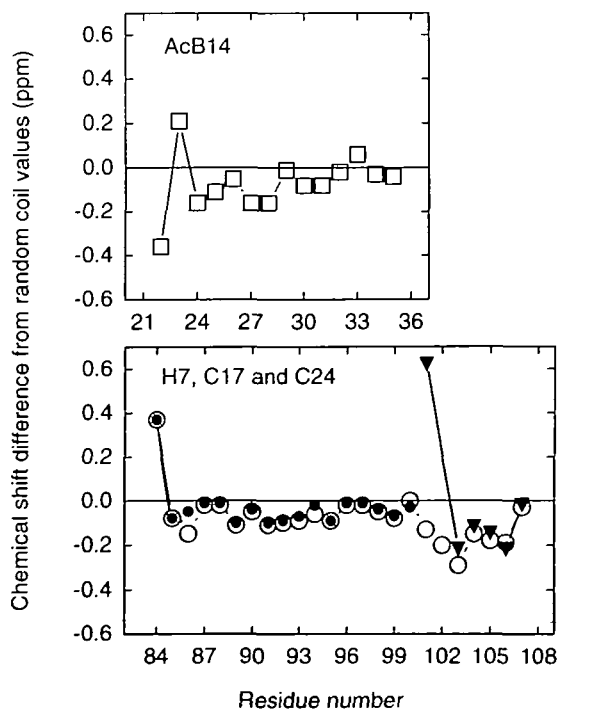

Fig. 4. **The chemical shift deviations from the random coil values are shown for the $C_\alpha H$ resonances of AcB14 (□), C17 (●), C24 (○), and H7 (▼).** Resonance assignments were done at pH 2.0 and 35°C.

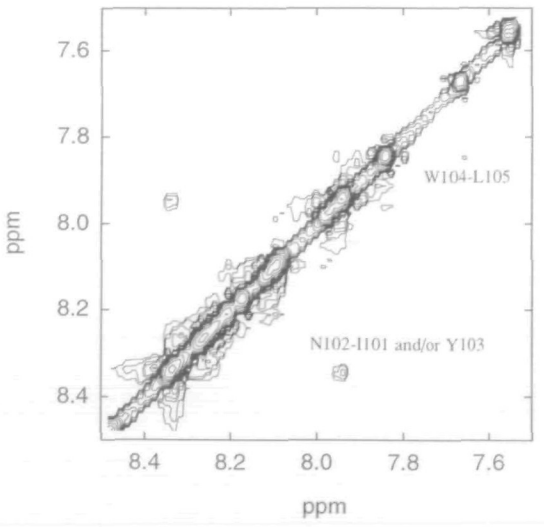

Fig. 5. **A portion of NOESY spectrum of C24 in 90% H_2O and 10% D_2O (pH 2.0, 35°C), showing d_{NN} connectivities.** The spectrum was acquired with the mixing time of 250 ms.

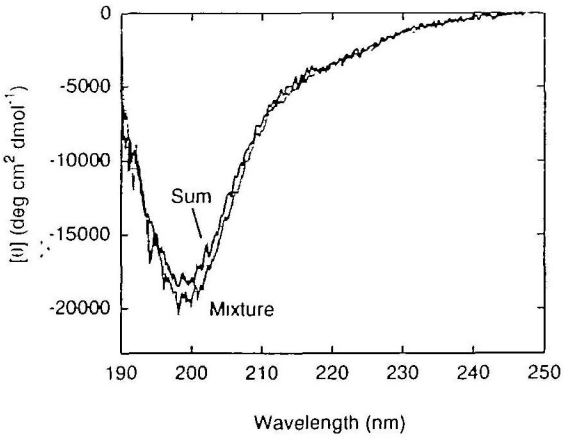

Fig. 6. **Comparison of the CD spectrum of mixture of AcB14 and C24 peptides with the sum of the spectra of individual peptides.**

peptide concentration should be as high as possible. Therefore, we investigated concentration dependence of the CD spectra of AcB14 and C24. The CD spectra of both peptides were independent of concentration at least below 0.5 mM. The spectrum of the peptide mixture was measured by mixing equal volumes of 0.876 mM AcB14 and 0.856 mM C24 solutions. As shown in Fig. 6, the spectrum of the peptide mixture to coincident with the sum of the spectra of individual peptides. This indicates that no structure is induced by mixing and that no detectable complex formation occurs at the peptide concentration used. By adding trifluoroethanol (TFE), both AcB14 and C24 assume helical structures, as judged by CD spectra (not shown). The ellipticities at 222 nm are −22,800 and −33,800 deg·cm²/dmol for AcB14 and C24, respectively, in 65% TFE. If 2% of the peptides associated with each other and they assumed helical structures, we could detect the CD spectrum change. Therefore, even though AcB14 and C24 can interact with each other in the absence of the other parts of the protein molecule, the binding constant is estimated to be lower than 50 M^{-1}.

DISCUSSION

In this paper, we have shown that the peptides encompassing the B or C helices of α-lactalbumin, which are known to be formed in the molten globule state, assume no detectable helical structures. Similar results have been observed for other proteins. The A, G, and H helices formed in the molten globule state of apomyoglobin are not stable in the isolated peptides (*15, 31-33*). The N- and C-terminal helices and the 60-75 helix, which are known to be formed in the molten globule of cytochrome *c*, do not also assume stable helix in the isolated peptides (*18, 34-36*). These results indicate that the helix in the molten globule is stabilized by some longer-range interactions. Our results show that this is also the case for the molten globule of bovine α-lactalbumin. Chyan *et al.* have mentioned that peptides corresponding to the isolated B and C helices of α-lactalbumin appear to be entirely unfolded in water, although they have not shown the detailed experimental results (*14*). Previous fragmentation studies of α-lactalbumin also showed that neither of the fragments 1-90 and 91-123 assumes an appreciable amount of helix (*37*).

The residues responsible for the helix stabilization in the molten globule have not yet been identified. A protein dessection study by Peng and Kim (*9*) has shown that the β-domain (residues 40-80) is not essential for the helix formation in the molten globule of α-lactalbumin. Kim and coworkers have also shown that the α-domain has a native-like backbone topology (*9, 10*). Therefore, the examination of the native conformation (*5-8*) should reveal which residues play a key role in stabilizing the B and C helices in the molten globule. The B and C helices do not make contact with each other in the native structure (Fig. 1). The 101-107 region is located between the B and C helices in the native structure, and the side chains of I101 and W104 are inserted into the space between the helices. In this study, however, the interaction of the C helix with the 101-107 region was not detected in AcC24, and neither was the direct interaction of the B helix with the C helix or the 101-107 region. Kuroda points out that the weak interaction between peptides is not detected by the simple mixing experiment of peptides (*36*). He has shown that the dimerization of peptides by disulfide bridges enhances the helicity of peptides through nonspecific interactions between the two helices. In intact α-lactalbumin, the B and C helices are linked by the intervening polypeptide chain (residues 36-83) and the disulfide bond between Cys28 and Cys111. These covalent linkings should increase the effective concentration of the B and C helices relative to each other. It has been shown, however, that disulfide bonds are not critical for the helix formation in the molten globule (*38, 39*). The effect of the intervening polypeptide chain can be estimated from the effective concentration of disulfide bond formation between Cys28 and Cys111 (*40*). Peng *et al.* have shown that the effective concentration of the Cys28-Cys111 disulfide bond is 1 mM in 7.5 M GdnHCl (*40*). Because the bimolecular binding constant between AcB14 and C24 is estimated to be lower than 50 M^{-1}, the failure to detect the complex formation of peptide in this study is thought to be not simply due to the bimolecular nature of the reaction. It is concluded, therefore, that the B

and C helices are not stabilized without long range interaction with some of the residues that are not included in the peptides used in this study.

In the native structure, the space between the B and C helices is occupied by the side chains of F8, L12, L15, L52, and F53. Because L52 and F53 are included in the β-domain which is known to be not essential for the helix formation in the molten globule (*9*), the importance of F8, L12, and L15 is suggested. The formation of the A helix (*5-11*) and the 3_{10} helix (*12-16*), which are amphipathic helices, is necessary for the side chains of F8, L12, and L15 to protrude in the same direction. In the molten globule, therefore, the helical structure is thought to be formed in the region 5-16. After this work was completed, the amide protons in the A helix and L12 were found to be strongly protected from exchange in the molten globule of recombinant human α-lactalbumin (*41*).

Recently, Yang *et al.* have synthesized four peptides spanning the entire sequence of hen egg white lysozyme, which is a homologous protein with α-lactalbumin, and investigated their structures (*42*). They have shown that the CD spectrum of the mixture of four peptides is similar to that of disulfide-reduced lysozyme and differs from that of transient folding intermediate. Their results indicate that, even if the entire sequence is present as peptides, the secondary structure of the early folding intermediate is not formed. However, because disulfide-reduced α-lactalbumin assumes the molten globule structure (*38, 39*) whereas disulfide-reduced lysozyme is essentially unfolded (*42*), it seems that the contribution of various factors to the stability of the secondary structure in the folding intermediate is different even among the homologous proteins. Whether the molten globule of α-lactalbumin can be mimicked by other peptides will be clarified in future work.

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