A Protein Dissection Study of a Molten Globule†

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ABSTRACT: Proteins have many distinct tertiary folds (Richardson, J. S. (1981) *Adv. Prot. Chem. 34,* 167–339). The term tertiary fold refers to the spatial organization of secondary structure elements (α-helices and β-strands). It is not known when, in the process of protein folding, a native tertiary fold emerges. Here, we show that the helical domain of human α-lactalbumin, in isolation, forms a molten globule with the same overall tertiary fold as that found in intact α-lactalbumin. Formation of this nativelike fold does not require extensive, specific side-chain packing. Our results suggest that much of the information transfer from one-dimension to three-dimensions has occurred at the molten globule stage of protein folding.

Molten globules have received substantial attention because they have been postulated to be general, early intermediates of protein folding (for reviews, see Ptitsyn, 1987, 1992; Kuwajima, 1989; Christensen & Pain, 1991; Haynie & Freire, 1993). The properties of molten globules include the following: (i) substantial secondary structure, comparable to that of the native protein; (ii) absence of well-defined tertiary packing; (iii) lack of a cooperative thermal unfolding transition; and (iv) compactness. Very little is known about the tertiary structures of molten globules; such information would clarify the role of these intermediates in the process of protein folding.

The best-studied molten globule is the low-pH form (A-state)[1] of α-lactalbumin (α-LA) (Kuwajima et al., 1976, 1985; Nozaka et al., 1978; Dolgikh et al., 1981, 1985; Ikeguchi et al., 1986; Gast et al., 1986; Baum et al., 1989; Xie et al., 1991; Alexandrescu et al., 1993). The following observations suggest that the α-helical domain forms the core of the α-LA molten globule. First, the molten globule of α-LA has essentially the same helix content as native α-LA (Kuwajima et al., 1976; Nozaka et al., 1978; Dolgikh et al., 1985). Second, NMR amide proton exchange and NOE studies suggest that at least a subset of the helices in native α-LA remains helical in the A-state (Baum et al., 1989; Alexandrescu et al., 1993). Finally, the α-helical domain forms early in the folding of hen egg-white lysozyme (Miranker et al., 1991; Radford et al., 1992), a protein that is structurally homologous to α-LA.

One approach for studying protein-folding intermediates is to remove regions that are thought to be unnecessary for the structure and properties of interest. We refer to this approach as "protein dissection". Protein dissection has been successfully applied to a number of proteins (Bierzynski et al., 1982; Vita et al., 1984; Dyson et al., 1985; Oas & Kim, 1988; Staley & Kim, 1990; Tasayco & Carey, 1992).

We have designed and constructed a single-chain, recombinant model of the α-helical domain of α-LA, called α-Domain (Figure 1). α-Domain consists of residues 1–39 and 81–123 of human α-LA, connected by a short linker of three glycines (the distance between the carbonyl of Gln 39 and the amide of Leu 81 is 7 Å in the crystal structure of α-LA). Cys 91, which forms an interdomain disulfide bond in α-LA, is changed to Ala to avoid unwanted thiol–disulfide reactions. α-Domain has 86 amino acids, including an N-terminal methionine as a result of expression in *Escherichia coli*. For simplicity, the α-LA numbering system is used for residues in α-Domain.

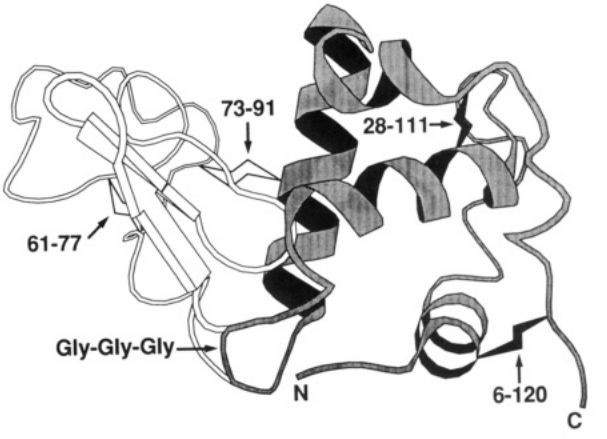

FIGURE 1: Schematic representation (Priestle, 1988) of α-LA. The α-helical domain contains all four α-helices in α-LA, whereas the β-sheet domain contains a small antiparallel β-sheet and several looplike structures (Acharya et al., 1989, 1991). The recombinant α-Domain (shaded) consists of residues 1–39 and 81–123 of α-LA plus a linker of three glycines and the substitution Cys 91 → Ala. α-LA has four disulfide bonds; two of them (6–120 and 28–111) are in the α-helical domain, one (61–77) is in the β-sheet domain, and one (73–91) connects the two domains. The two disulfide bonds in α-Domain^{ox} (6–120 and 28–111) are shown in black.

MATERIALS AND METHODS

Recombinant α-Domain was expressed in *E. coli* from a synthetic gene driven by the T7 promoter, using the cloning vector pAED4 (Doering, 1992). Cells were lysed by sonication in buffer containing 50 mM Tris, 25% sucrose, and 1 mM EDTA, pH 8. The insoluble fraction was washed with the same buffer and with buffer containing 20 mM Tris, 1% Triton X-100, and 1 mM EDTA, pH 8. The resulting pellet (inclusion bodies) was solubilized in 50 mM Tris, 50 mM NaCl, 8 M urea, 5 mM EDTA, and 0.1 M DTT, pH 8.5, and loaded onto

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[1] Abbreviations: α-LA, α-lactalbumin; A-state, low-pH form of α-LA; α-Domain, a recombinant model of the isolated helical domain of human α-LA containing residues 1–39 and 81–123 connected by a linker of three glycine residues and containing the substitution Cys 91 → Ala; CD, circular dichroism; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; HPLC, high-performance liquid chromatography; GuHCl, guanidine hydrochloride; DTT, dithiothreitol; TFA, trifluoroacetic acid.

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a fast-flow DEAE-Sepharose (Sigma) column equilibrated with 50 mM Tris, 50 mM NaCl, 4 M urea, 1 mM EDTA, and 5 mM DTT. The column was eluted with a linear gradient of NaCl (final concentration, 0.5 M) in 4 M urea. The partially purified α-Domain was diluted to $\sim$0.5 mg/mL with 4 M urea and dialyzed extensively at 4 °C against a buffer containing 10 mM Tris, 50 mM NaCl, 1 mM $CaCl_2$, 2.5 mM reduced glutathione, and 0.5 mM oxidized glutathione, pH 8.5, during which oxidative refolding occurs. The oxidized α-Domain, denoted α-Domain^{ox}, was purified by reverse-phase HPLC. The identity of α-Domain^{ox} was confirmed by laser desorption mass spectrometry (Finnigan LASERMAT; calculated molecular weight, 9663 Da; observed, 9662–9665 Da).

The disulfide-bond pattern in α-Domain^{ox} was assigned by digestion with pepsin (Carr et al., 1991) followed by analytical reverse-phase HPLC. Two peptide fragments with elution times that are shifted in the presence of DTT were isolated. N-terminal sequencing, amino acid analysis, and mass spectrometry indicated that these fragments correspond to (i) Phe 3–Leu 11/Trp 118–Leu 123 (disulfide 6–120) and (ii) Ile 27–Phe 31/Trp 104–Thr 112 (disulfide 28–111).

Sedimentation equilibrium studies were performed at 4 °C on a Beckman XL-A analytical ultracentrifuge, using an An-60 Ti rotor at speeds of 28 and 33 krpm for α-Domain^{ox} or 25 and 30 krpm for human α-LA. The protein solutions ($\sim$1 mg/mL) were dialyzed against buffers containing 10 mM Tris and 0.5 mM EDTA, pH 8.5, for α-Domain^{ox} and 10 mM Tris and 1 mM $CaCl_2$, pH 8.5, or 10 mM HCl, pH 2, for human α-LA. Three dilutions with final protein concentrations of 60–80, 20–27, and 6–9 μM were run in Beckman 6-sector cells. The apparent molecular weights were calculated for each sector using the program NONLIN (courtesy of M. L. Johnson and J. Lary) with a partial specific volume of 0.736 for α-Domain^{ox} and 0.729 for human α-LA (Laue et al., 1992).

CD spectra were recorded at 0 °C on an Aviv 62DS circular dichroism spectrometer equipped with a thermoelectric temperature controller; 10, 20, 40, and 80 μM protein in a 1-mm path length cuvette were used for far-UV studies (1.5-nm bandwidth), and 40 μM protein in a 10-mm cuvette was used for near-UV studies (5-nm bandwidth). Samples at pH 8.5 contained 10 mM Tris and 0.5 mM EDTA for α-Domain^{ox} or 10 mM Tris and 1 mM $CaCl_2$ for human α-LA. In addition, the sample of reduced α-Domain contained 2 mM DTT. Samples at pH 2 contained no buffer; the pH was adjusted with 0.1 M HCl. Unfolded α-Domain^{ox} was studied in 6 M GuHCl, 0.1 M Tris, and 0.5 mM EDTA, pH 8.5. At pH 2, both α-Domain^{ox} and human α-LA exhibited a higher ellipticity in the far-UV region than at pH 8.5, for reasons that are not well understood. The mean residue ellipticities of α-Domain^{ox}, [6–28; 111–120], [6–111; 28–120], and reduced α-Domain did not depend on protein concentration from 10 to 80 μM. For determination of the temperature dependence at 222 nm, 10 μM protein in a 10-mm cuvette was used. All temperature traces were over 80% reversible, and the qualitative features in the forward and reverse directions were the same. The protein concentrations were determined by absorbance at 280 nm in 6 M GuHCl (Edelhoch, 1967).

Fluorescence spectra were recorded at 4 °C on an ISS PC spectrofluorimeter operating in ratio mode. The excitation wavelength was 278 nm. Both the excitation and emission slits were set to 1 mm. The samples contained 10 μM protein in the same buffers used for CD studies.

NMR spectra were collected at 4 °C on a Bruker AMX 500-MHz NMR spectrometer. Free induction decays were averaged over 1024 scans with 4 K complex data points. The samples contained $\sim$80 μM protein in D_2O; additionally, pH 8.5 samples contained 0.5 mM EDTA (deuterated) for α-Domain^{ox} and 1 mM $CaCl_2$ for α-LA. All proteins are monomeric at this concentration, as determined by sedimentation equilibrium. Chemical shifts were determined relative to an internal (trimethylsilyl)propionate (TMSP) standard, corrected for the pH-dependence of the TMSP chemical shift (DeMarco, 1977).

Dynamic light-scattering studies were performed at 4 °C on a Brookhaven Instrument BI-2030 laser light-scattering apparatus equipped with an Ar^+ laser operating at 488 nm. Autocorrelation functions were sampled at 0.5- or 1-μs time intervals, for a total accumulation time of 15–60 min. Experiments were carried out in the same buffers as used for CD studies. Protein solutions were passed through a 100-kDa Centricon (Amicon) to remove trace amounts of large aggregates and dust. The protein concentrations were $\sim$80 μM ($\sim$120 μM for unfolded). Hydrodynamic diameters were calculated by the cumulant method (Koppel, 1972), with polydispersity in the range of 0.2–0.3. A refractive index of 1.333 and a viscosity of 1.567 centipoise were used for dilute aqueous solutions at 4 °C. A refractive index of 1.465 and a viscosity of 2.23 centipoise were used for 6 M GuHCl solutions. These values were determined with a refractometer (Bausch & Lomb) and a capillary viscometer (Cole-Parmer).

Non-native disulfide-bonded species of α-Domain were purified from a mixture obtained by complete air oxidation of reduced α-Domain in 2–6 M GuHCl. The purity of each species is >90%, as judged by analytical HPLC. The disulfide-bond patterns for non-native species were assigned by proteolysis experiments. The following peptides have been identified. For [6–28; 111–120]: (i) Phe 3–Leu 11/Ile 27–Phe 31 (disulfide 6–28) and (ii) Trp 104–Leu 123 (disulfide 111–120). For [6–111; 28–120]: (i) Phe 3–Leu 11/Trp 104–Thr 112 and Phe 3–Leu 11/Trp 104–Gln 117 (disulfide 6–111) and (ii) Ile 27–Phe 31/Trp 118–Leu 123 (disulfide 28–120).

Disulfide-exchange experiments were carried out at room temperature in an anaerobic glovechamber (Coy Laboratory Products). The native buffer contained 10 mM Tris and 0.5 mM EDTA, pH 8.5; the denaturing buffer contained 0.1 M Tris, 6 M GuHCl, and 0.5 mM EDTA, pH 8.5. Disulfide exchange was initiated by adding α-Domain to buffers containing 100 μM *N*-(2,4-dinitrophenyl(DNP))-L-cysteine and 10 μM *N*,*N*-bis(2,4-DNP)-L-cystine (Sigma). (The DNP-labeled cysteine was obtained by reducing the DNP-labeled cystine with DTT followed by HPLC purification.) The final concentration of α-Domain was 5 μM. The 2,4-DNP group served as a spectroscopic label (340 nm) for identifying mixed disulfide species. After $\sim$10 h of equilibration, samples were quenched by adding formic acid to a final concentration of 10% (v/v) followed by analytical HPLC using a H_2O–acetonitrile gradient (0.09%/min increase of acetonitrile) with 0.1% TFA. Identical results were obtained using a redox buffer with 100 μM reduced glutathione and 10 μM oxidized glutathione. The errors in peak quantitation were estimated to be less than 1%. The redox conditions were chosen such that the mixed disulfide population at equilibrium was small; none of the HPLC peaks discussed in this paper showed significant absorbance at 340 nm. In addition, these peaks do not contain free thiols, as the elution times of these peaks did not change if 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Ellman, 1959) was added (1 mM final concentration) before the acid quench, with or without the addition of 6 M GuHCl.

Table 1: Summary of Physical Properties of α-Domain^{ox} and α-LA at 4 °C

	apparent molecular weight (kDa)	hydrodynamic diameter (Å)	mean residue ellipticity, 222 nm (10^3 deg cm^2/dmol)	mean residue ellipticity, 270 nm (deg cm^2/dmol)	tryptophan fluorescence maximum (nm)	cooperative thermal transition	substantial chemical shift dispersion
native α-LA (pH 8.5, 1 mM $CaCl_2$)	13.6	35.3	−7.9	−220	326	yes	yes
molten globule α-LA (pH 2.0)	13.6	37.5	−12.5	−50	336	no	no
α-Domain^{ox} (pH 8.5, 0.5 mM EDTA)	10.1	36.8	−11.7	−20	338	no	no
α-Domain^{ox} (pH 8.5, 6 M GuHCl)		~77	~−1	−50	345		

RESULTS

A species denoted α-Domain^{ox}, which has the same disulfide bonds (6–120 and 28–111) as native α-LA, exhibits properties of a molten globule under a broad range of conditions. Some physical properties of α-Domain^{ox} are summarized in Table 1. Since molten globules have a tendency to aggregate (Goto & Fink, 1989; Kuwajima, 1989), it is especially important to establish that one is studying a monomeric species. Sedimentation equilibrium studies show that α-Domain^{ox} is >90% monomeric at concentrations below 100 μM at pH 8.5 (10 mM Tris, 0.5 mM EDTA) with no additional salt. Nonideality was not observed at protein concentrations in the range of 5–30 μM, and the residuals revealed no systematic deviation. At the highest protein concentration (>60 μM), the apparent molecular weight decreases by 5–10%, probably as a result of electrostatic interaction with the counterion gradient (Williams et al., 1958). All studies of α-Domain^{ox} (including NMR) were carried out under these conditions. The CD and NMR spectra of α-Domain^{ox} do not change when EDTA is replaced by 1 mM $CaCl_2$. For comparison, intact human α-LA was studied both in the A-state (pH 2) and in native conditions (pH 8.5, 1 mM $CaCl_2$).

Far-UV CD spectra indicate that α-Domain^{ox} has substantial α-helical secondary structure (Figure 2A), as expected for a molten globule. These spectra suggest that essentially all of the helices in α-LA are preserved in α-Domain^{ox}. The near-UV CD spectrum of α-Domain^{ox} lacks the characteristic features seen in spectra of native α-LA but is similar to the spectrum of the A-state of α-LA (Figure 2B), suggesting disrupted side-chain packing. (α-Domain^{ox} retains two of the three tryptophans and three of the four tyrosines in human α-LA.) The temperature dependence of the CD signal for α-Domain^{ox} parallels that of the A-state of α-LA; neither exhibits a cooperative thermal transition (Figure 2C,D). As with the A-state of α-LA, the fluorescence maximum of α-Domain^{ox} is red-shifted compared to the maximum of native α-LA (Table 1), presumably because the tryptophans are more solvent exposed than in the native protein.

The proton NMR spectrum of α-Domain^{ox} resembles the spectrum of the A-state of α-LA (Figure 3). Both spectra exhibit line broadening and considerably less chemical shift dispersion than is observed for native α-LA, suggesting that α-Domain^{ox} has a slowly fluctuating structure and that the side chains in α-Domain^{ox} are not in well-defined conformations (Dolgikh et al., 1985; Baum et al., 1989; Alexandrescu et al., 1993).

Dynamic light-scattering studies show that α-Domain^{ox} is compact, as compared to the unfolded form in 6 M GuHCl (Table 1). With a spherical approximation, the hydrodynamic diameter of α-Domain^{ox} is ~20% larger than the value predicted if it were as compact as native α-LA. This result is consistent with the hydrodynamic properties observed for other molten globules (Gast et al., 1986; Goto et al., 1990).

Hypotheses about the structures of molten globules often fall into two categories (for reviews, see Kuwajima, 1989; Ptitsyn, 1992): (i) a nonspecific assembly of secondary structure elements or (ii) an ensemble of slightly different structures sharing a common tertiary fold. For proteins containing disulfide bonds, the tertiary fold can be probed by studying the specificity of disulfide-bond formation.

We examined each of the three possible forms of α-Domain with two disulfide bonds. Non-native disulfides force peptide segments that are distant in the native structure to be close in space, thereby imposing a non-native tertiary fold. Far-UV CD spectra show that both of the species with non-native disulfide bonds, [6–28; 111–120] and [6–111; 28–120], have considerably less helical secondary structure than α-Domain^{ox} (Figure 4). The helix content of α-Domain^{ox} is higher than that of reduced α-Domain, which in turn is higher than the helix content of either of the non-native species.

The relative stability of different two-disulfide species at equilibrium can be determined by disulfide-exchange experiments (Figure 5). Because the native disulfide bonds in α-Domain are between cysteines that are distant in sequence, a random-walk model predicts that only 7% of the two-disulfide species should have native disulfides (Kauzmann, 1959; Snyder, 1987). Instead, under native conditions, 90% of the two-disulfide species are α-Domain^{ox}. As a control, in 6 M GuHCl, only 8% of the two-disulfide species have native disulfide bonds, in excellent agreement with the prediction of the random-walk model. In both conditions, identical ratios of the two-disulfide species are obtained using either α-Domain^{ox} or reduced α-Domain as the starting material. We conclude that, under native conditions, a nativelike tertiary fold is the most stable fold for α-Domain.[2]

DISCUSSION

Our results demonstrate that α-Domain^{ox} is a molten globule with a nativelike tertiary fold (i.e., the spatial arrangement of helices is similar to that of native α-LA). Complementary results from hydrogen–deuterium exchange studies have shown that the helices in molten globules, including the A-state of α-LA, are formed by the same, or nearly the same, residues that form helices in the corresponding native proteins (Baum et al., 1989; Hughson et al., 1990; Jeng et al., 1990; Alexandrescu et al., 1993). Taken together, these results suggest that molten globules are stabilized by native interactions and contain structures that are closer to expanded native proteins than to nonspecific, collapsed polypeptides.

Previous studies of disulfide exchange in the molten globule of α-LA suggested that, although there were some restrictions, a variety of disulfide pairings were favored (Ewbank &

[2] The free-energy differences between conformations with native and non-native tertiary folds can be estimated from the ratios of the equilibrium populations between α-Domain^{ox} and each of the non-native disulfide species under native versus denaturing conditions; the results are 2.9 and 1.8 kcal/mol favoring the native disulfide species for [6–28; 111–120] and [6–111; 28–120], respectively. These values are comparable to the free energy of unfolding the molten globule of α-LA (Kuwajima et al., 1976; Ikeguchi et al., 1986b).

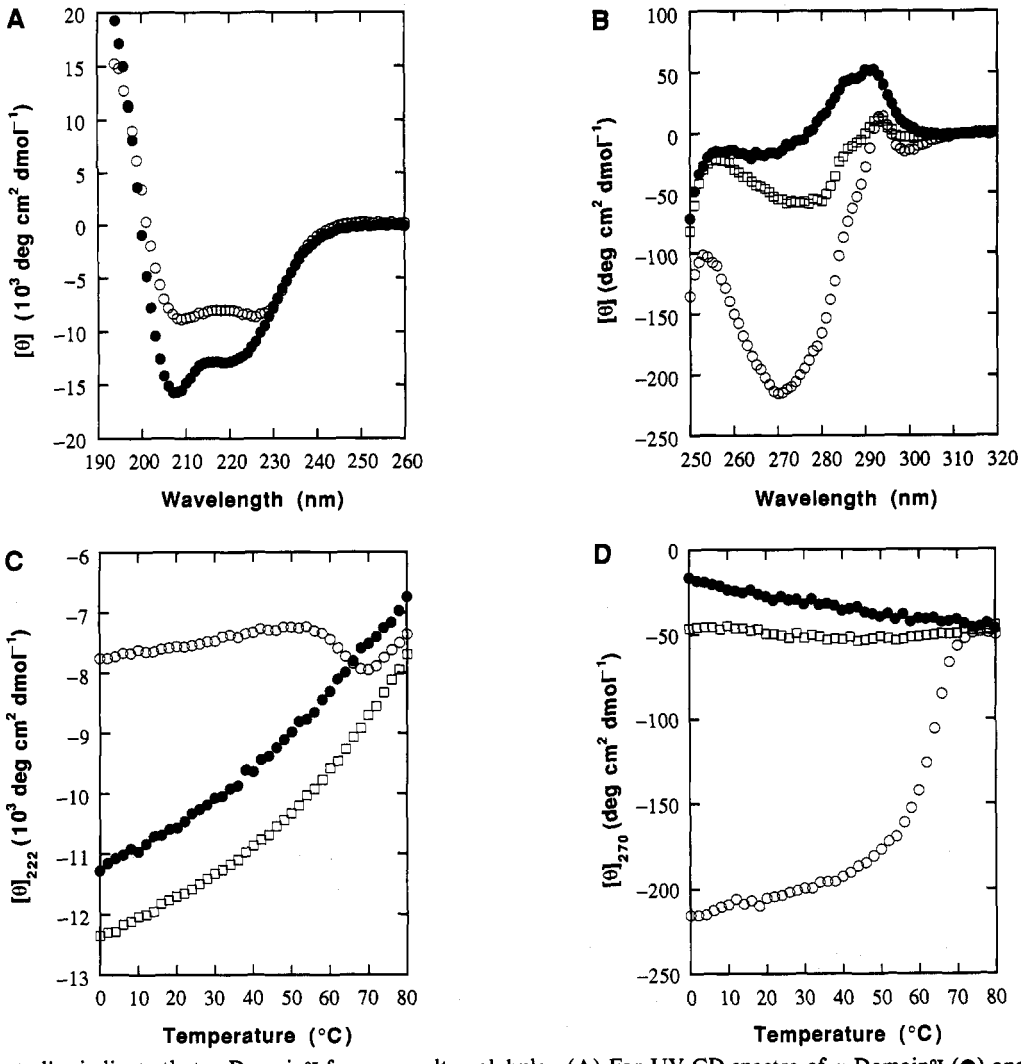

FIGURE 2: CD studies indicate that α-Domain^{ox} forms a molten globule. (A) Far-UV CD spectra of α-Domain^{ox} (●) and native α-LA (○) at pH 8.5. The ratio of the mean residue ellipticities at 222 nm of α-Domain^{ox} and α-LA indicates that the two molecules have approximately the same number of residues in an α-helical conformation. (B) Near-UV CD spectra of α-Domain^{ox} at pH 8.5 (●), native α-LA at pH 8.5 (○), and molten globule α-LA at pH 2 (□). α-Domain^{ox} retains two of the three tryptophans and three of the four tyrosines in human α-LA. (C) Temperature dependence of the CD signal at 222 nm and (D) at 270 nm for α-Domain^{ox} at pH 8.5 (●), native α-LA at pH 8.5 (○), and molten globule α-LA at pH 2 (□).

Creighton, 1991, 1993). One factor to be considered in the interpretation of these results, in light of our current observations, is that the molten globule of α-LA may have an α-helical domain with a nativelike fold but a relatively unstructured β-sheet domain. More work is needed to determine if the molten globule of α-LA includes both ordered and disordered regions.

The preference for native disulfides does not imply a rigid structure. The disulfide constraints still allow for substantial flexibility, including multiple side-chain conformations and small adjustments of helix crossing angles. Indeed, we refer to α-Domain^{ox} as a molten globule, rather than a destabilized native species, because even with native disulfide bonds, α-Domain^{ox} lacks a native near-UV CD spectrum, lacks chemical shift dispersion in NMR studies, and is less compact than native α-LA.

Short peptide fragments, including those corresponding to individual helices in α-LA (Alexandrescu et al., 1993), generally do not fold into stable structures in aqueous solution (Wright et al., 1988; Kim & Baldwin, 1990). By contrast, the helices in α-Domain^{ox} are stabilized cooperatively by forming a nativelike tertiary fold, even though folding does not proceed beyond the molten globule stage. In addition, we find that non-native tertiary folds, imposed by non-native disulfides, decrease the helix content in α-Domain. Thus, formation of a high level of secondary structure in α-Domain is linked to formation of a nativelike tertiary fold.

Remarkably, the nativelike tertiary fold in the α-Domain^{ox} molten globule is formed without extensive, specific side-chain packing. It is possible that a few side-chain interactions, yet to be discovered, play a crucial role in determining the tertiary fold of this molten globule. Alternatively, the amino acid sequence of a protein could specify the tertiary fold in a manner that does not depend on the details of side-chain packing (Behe et al., 1991). In the latter case, properties such as the pattern of hydrophobic and hydrophilic residues, side-chain volumes, and secondary structure propensities are probably the important determinants of tertiary folds (e.g., Lau & Dill, 1990; Bowie et al., 1991). This would imply that the native fold of a protein could be predicted without detailed knowledge of tertiary packing interactions.

α-Domain^{ox} is an example of a compact, autonomous folding unit (Wetlaufer, 1973), sufficient for formation of a molten globule with properties similar to the A-state of α-LA. In the folding of multidomain proteins, individual domains often fold independently (Jaenicke, 1991). Our results suggest that some

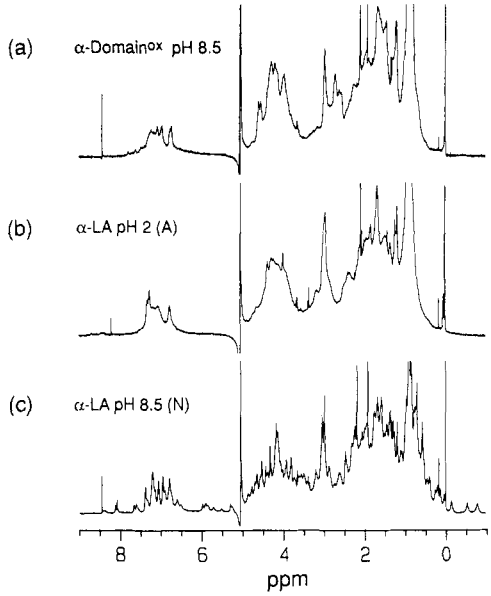

FIGURE 3: ^{1}H NMR spectra. The spectra of α-Domain^{ox} are similar to those of the A-state of α-LA and lack the chemical shift dispersion observed in native α-LA: (a) α-Domain^{ox} at pH 8.5, (b) molten globule α-LA at pH 2, and (c) native α-LA at pH 8.5.

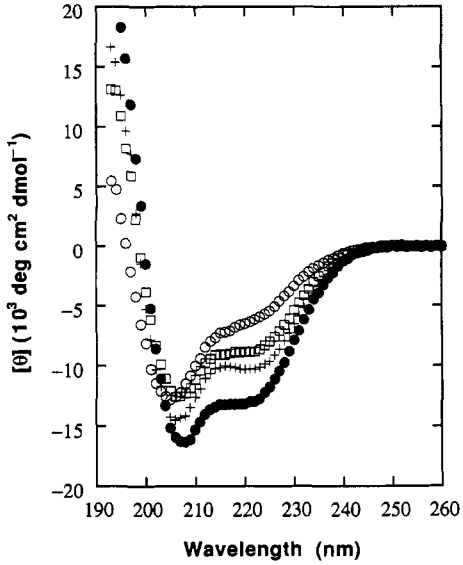

FIGURE 4: Far-UV CD spectra. α-Domain^{ox} (●), reduced α-Domain (+), [6–28; 111–120] (○), and [6–111; 28–120] (□). The spectra indicate that non-native tertiary folds, imposed by non-native disulfide bonds, significantly decrease the helix content of α-Domain.

of these folded domains in the folding intermediates of multidomain proteins may correspond to molten globules with nativelike tertiary folds (see, also, Goldberg et al., 1990).

Finally, even though they are equilibrium intermediates, molten globules appear to show characteristic properties of early kinetic folding intermediates (Kuwajima, 1989; Ptitsyn, 1992; Baldwin, 1993). Early formation of a nativelike tertiary fold within a molten globule intermediate will lead to a vast reduction of the conformational space to be searched at later stages of folding. At the same time, the potential energy surface near the molten globule state, which lacks extensive side-chain packing, should be smoother and shallower than that near the native state. Thus, the molten globule provides an approximate solution to the protein-folding problem while granting enough flexibility to make kinetic traps less likely. Our results, combined with other studies of molten globules, suggest that the process of protein folding depends on the

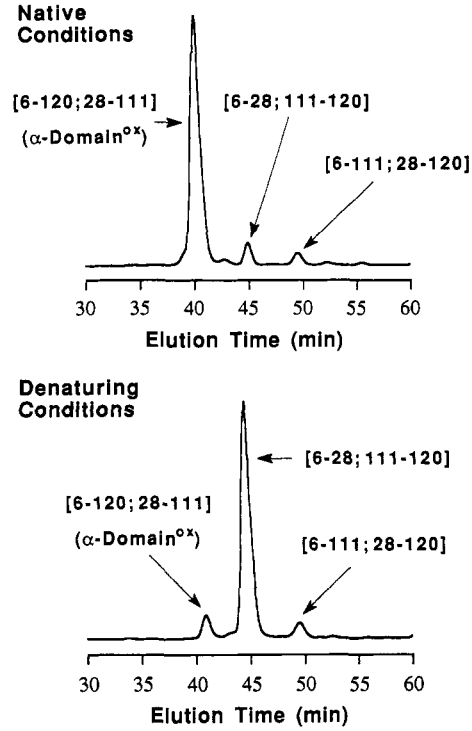

FIGURE 5: Disulfide exchange in α-Domain analyzed by HPLC. Under native conditions, the ratio of α-Domain^{ox}:[6–28; 111–120]:[6–111; 28–120] is 90:6:4. Under denaturing conditions (6 M GuHCl), the ratio of α-Domain^{ox}:[6–28; 111–120]:[6–111; 28–120] is 8:85:7. For comparison, the ratio of α-Domain^{ox}:[6–28; 111–120]:[6–111; 28–120] for the random-walk model is 7:88:5, where the probability of an intramolecular disulfide formation is proportional to $n^{-3/2}$ and $n-1$ is the number of intervening non-cysteine residues in the loop (Kauzmann, 1959; Snyder, 1987).

following order of events (Ptitsyn, 1991): (i) early onset of the native tertiary fold and (ii) late consolidation of side-chain packing. Accordingly, experimental and theoretical studies of protein folding might be simplified by focusing individually on each of these events.

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