Unfolding and Refolding Pathways of a Major Kinetic Trap in the Oxidative Folding of α-Lactalbumin†

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ABSTRACT: α-Lactalbumin (αLA)-IIIA is a major kinetic intermediate present along the pathways of reductive unfolding and oxidative folding of bovine α-lactalbumin (αLA). It is a three-disulfide variant of native αLA lacking Cys^{6}−Cys^{120} at the α-helical domain. Stability and the unfolding/refolding mechanism of carboxymethylated αLA-IIIA have been investigated previously by stop-flow circular dichroism (CD) and fluorescence spectroscopy. A stable intermediate compatible with molten globule was shown to exist along the pathways of unfolding-refolding of αLA-IIIA [Ikeguchi et al. (1992) *Biochemistry 31,* 16695−12700; Horng et al. (2003) *Proteins 52,* 193−202]. We investigate here the unfolding−refolding pathways and conformational stability of αLA-IIIA using the method of disulfide scrambling with the following specific aims: (a) to isolate and characterize the observed stable molten globule, (b) to analyze the heterogeneity of folding−unfolding intermediates, (c) to elucidate the disulfide structure of extensively unfolded isomer of αLA-IIIA, and (d) to clarify the relative conformational stability between αLA-IIIA and αLA. Two scrambled isomers, designated as X-αLA-IIIA-**c** and X-αLA-IIIA-**a** (X stands for scrambled), were isolated under mild and strong denaturing conditions. Their disulfide structures, CD spectra, and manners of refolding to form the native αLA-IIIA were analyzed in this report. The results are consistent with the notion that X-αLA-IIIA-**c** and X-αLA-IIIA-**a** represent a partially unfolded and an extensively unfolded isomers of native αLA-IIIA, respectively. The unfolding−refolding pathways of αLA-IIIA are elaborated and compared with that of intact αLA. These results display new insight into one of the most extensively studied molecules in the field of protein folding and unfolding.

α-Lactalbumin (αLA)[1] is a 14 kDa protein the native structure of which is differentiated by two domains (the α-helical domain and the *β*-domain) bound by four disulfide bridges. This protein represents one of the most widely studied models in the fields of protein folding and unfolding. αLA has become a paradigm for evaluating the properties of stable partially folded proteins (*1*−*5*), since it forms a stable molten globule intermediate under a variety of mildly denaturing conditions. Most of the approaches that report the α-LA folding and unfolding processes involve experimental conditions performed at low pH (*6*−*8*), elevated temperature (*9*), or mild concentration of denaturant (*10*−*12*). Not only have these technical approaches been applied to study the wild-type αLA, they have also been used in a number of folding and unfolding studies performed with αLA mutants. These mutants, mostly with the wild-type cysteine modified to obtain variants with zero to three disulfide bridges, represent a valuable resource to better comprehend the structural characteristics of the αLA molten globule, as well as other folding intermediates. Also, the study of these variant forms has provided valuable insight into the wild-type α-LA folding process and the stability of the molecule. Examples of these mutants include αLA[all-Ala] (zero disulfides) (*13*), αLA[28−111] (one disulfide) (*14*), and αLA(α) and αLA(*β*) (two disulfides) (*15*, *16*).

αLA-IIIA is a three-disulfide variant of intact αLA. It is a major intermediate and kinetic trap present along the pathways of reductive unfolding and oxidative folding of αLA (*17*, *18*). αLA-IIIA contains two free cysteines (Cys^{6} and Cys^{120}) and three native disulfide bridges of αLA. Two of them are located at the *β*-subdomain (Cys^{61}−Cys^{77} and Cys^{73}−Cys^{91}), and the third bridge is located within the α-helical domain of the molecule (Cys^{28}−Cys^{111}). Carboxymethylated αLA-IIIA is also known as 2CM-3SS-BLA or 3SSCOOH-αLA in previous studies (*19*, *20*).

αLA-IIIA represents a useful model for further analysis of the property of molten globule. Although αLA-IIIA exhibits decreased conformational stability due to the absence of the Cys^{6}−Cys^{120} disulfide bond, its native conformation is essentially identical to that of intact αLA (*20*). The conformational stability, equilibrium unfolding, and kinetic refolding of carboxymethylated αLA-IIIA have been investigated by stop-flow circular dichroism (CD) and fluorescence spectroscopy. A stable intermediate compatible with molten globule state of αLA was shown to exist in both pathways of unfolding and refolding of αLA-IIIA (*19*). These

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[1] Abbreviations: GdmCl, guanidine chloride; α-LA, bovine α-lactalbumin; HPLC, high-pressure liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight.

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studies were performed under conditions that allow the three native disulfide bonds of αLA-III to remain intact. Despite previous effort, heterogeneity of unfolding and refolding intermediates of αLA-IIIA has not been quantitatively determined. Most importantly, the observed stable intermediate (*19*) has yet to be isolated and characterized. Isolation of this αLA-III-associated intermediate is important because it will allow further comprehension of the elusive structure of molten globule.

In this report, we have investigated the unfolding and refolding pathways of αLA-III using the technique of "disulfide scrambling" (*21*, *22*) with the specific aim to isolate and characterize the stable intermediate detected previously by the method of "disulfide intact" denaturation. The method of "disulfide scrambling" permits a denatured protein to be trapped, kinetically or thermodynamically, as a mixture of diverse scrambled isomers, which are amenable to separation by HPLC and further structural analysis.

MATERIALS AND METHODS

Materials. Calcium-depleted bovine αLA (L-6010) was used throughout this study and was obtained from Sigma. The protein was further purified by HPLC and was shown to be more than 97% pure. Urea, guanidine hydrochloride, acetonitrile, and 2-mercaptoethanol with purities greater than 99% were also purchased from Sigma.

Preparation of αLA-IIIA. αLA-IIIA can be prepared from oxidative folding of reduced αLA or from mild reduction of native αLA (*17*). Native αLA (2 mg/mL) was reduced in the Tris-HCl buffer (0.1 M, pH 8.4) containing dithiothreitol (0.3 mM) and $CaCl_2$ (5 mM). The reaction was allowed to proceed for 10 min at 23 °C. Under these conditions, Cys^6−Cys^{120} of the native αLA was selectively and quantitatively reduced to generate αLA-IIIA, which was purified by HPLC. The fraction containing pure αLA-IIIA was freeze-dried and derivatized with 20 mM iodoacetic acid for another 30 min. Carboxymethylated αLA-IIIA was further purified by reversed-phase HPLC, verified by MALDI mass spectrometry, and used for studies described in this report. This three-diuslfide variant of αLA is also known as 2CM-3SS-BLA (2-carboxymethylated three-disulfide bovine α-lactalbumin)(*19*) and 3SSCOOH-αLA (*20*).

Denaturation and Unfolding of αLA-IIIA. Chemical denaturation of αLA-IIIA was performed in a thermodynamic fashion. Carboxymethylated αLA-IIIA (0.5 mg/mL) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing 0.2 mM of 2-mercaptoethanol and selected concentrations of denaturants (urea, GdmCl, or acetonitrile). The reaction was typically performed at 23 °C for 20 h to ensure the equilibrium of the unfolding reactions. The unfolded protein was quenched with an equal volume of 4% aqueous trifluoroacetic acid and directly analyzed by reverse-phase HPLC. Thermal denaturation was carried out in a kinetic manner. αLA-IIIA (0.5 mg/mL) was dissolved in Tris-HCl buffer (0.1 M, pH 8.4) containing 0.1 mM 2-mercaptoethanol and allowed to unfold at 65 °C for a period of up to 60 min. To monitor the kinetics of unfolding, aliquots of the sample were removed at different time points, quenched with an equal volume of 4% aqueous trifluoroacetic acid, and directly analyzed by reverse-phase HPLC.

Structural Analysis of Scrambled Isomers of αLA-IIIA. Fractions of X-αLA-IIIA-**a** and X-αLA-IIIA-**c** (∼15 μg) were isolated from selected unfolding conditions, freeze-dried, and treated with 1.5 μg of thermolysin (Sigma, P-1512) in 30 μl of *N*-ethylmorpholine/acetate buffer (50 mM), pH 6.4. Digestion was carried out at 37 °C for 16 h. Peptides were then isolated by HPLC and analyzed by both mass spectrometry and Edman sequencing to identify the disulfide-containing fragments.

Refolding of Scrambled Isomers of αLA-IIIA. X-αLA-IIIA-**a** and X-αLA-IIIA-**c** were allowed to refold to form the native αLA-IIIA in the Tris-HCl buffer (0.1 M, pH 8.4) containing 0.1 mM 2-mercaptoethanol. Folding reaction was quenched at different time points by acidification with an equal volume of 4% aqueous trifluoroacetic acid and directly analyzed by reverse-phase HPLC.

Amino Acid Sequencing, Mass Spectrometry and CD Spectroscopy. Amino acid sequences of disulfide-containing peptides were analyzed by automatic Edman degradation using a Perkin-Elmer Procise sequencer (model 494) equipped with an on-line PTH-amino acid analyzer. The molecular masses of disulfide-containing peptides were determined by MALDI-TOF mass spectrometry (Perkin-Elmer Voyager-DE STR) using 2,5-dihydroxybenzoic acid as matrix. Molecular masses of analyzed peptides were calibrated by the following standards: bradykinin fragment (residues 1−7) (MH^+ 757.3997), synthetic peptide P14R (MH^+ 1533.8582), ACTH fragment (residues 18−39) (MH^+ 2465.1989), and insulin oxidized B-chain (MH^+ 3494.6513). Spectra of CD of isomers of αLA and αLA-IIIA were measured at the protein concentration of 0.56 mg/mL in the Tris-HCl buffer (20 mM, pH 7.4) in a 100 μL cuvette (1 mm light pass) using a Jasco J-715 spectropolarimeter. The protein concentration was determined according to the Bradford method (Bio-Rad kit). The individual isomers were purified by HPLC, as described. A full scan was performed for each sample from 255 to 195 nm. The helicity of each sample was transformed from the mean residue ellipticity at 222 nm.

Plotting of the Thermodynamic Denaturation Curves of αLA-IIIA. Denaturation of αLA-IIIA is defined by the conversion of the native structure to scrambled isomers. Therefore the denaturation curve of αLA-III was plotted as the fraction (%) of the native αLA-III converted to the scrambled isomers (there are 14 possible isomers). Quantitative analysis of the relative yield of the scrambled and native isomers was based on the integration of HPLC peak areas. The thermodynamic denaturation curve of αLA was derived from those that have attained the end point (equilibrium) of denaturation under increasing concentrations of a selected denaturant.

RESULTS

Denaturation and Unfolding of αLA-IIIA by Urea and GdmCl. A denatured disulfide protein may adopt varied extents of unfolding. Denaturation and unfolding are therefore two distinct terms. When the method of disulfide scrambling is used (*21*, *23*), it is possible to observe and follow simultaneously the process of denaturation and unfolding of αLA-IIIA. The extent of denaturation of αLA-IIIA is defined and measured by the simple conversion of the native structure to non-native structures (scrambled isomers). There are 14 scrambled isomers of denatured αLA-IIIA versus one isomer of the native αLA-IIIA. Unfolding

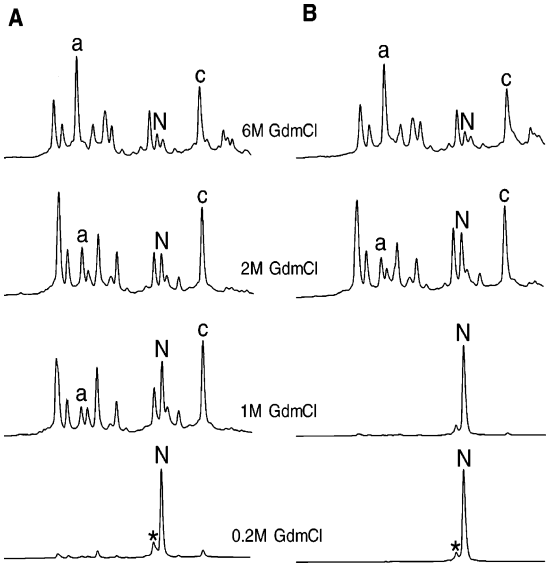

FIGURE 1: Denaturation and unfolding of αLA-IIIA by different concentrations of GdmCl in the presence of thiol catalyst. Reactions were carried out both in the absence (A) and in the presence (B) of $CaCl_2$ (5 mM). Native αLA-IIIA (0.5 mg/mL) was denatured in Tris-HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.1 mM) and the indicated concentrations of GdmCl. All reactions were carried out at 23 °C for 24 h. The denatured sample was quenched with an equal volume of 4% aqueous trifluoroacetic acid and analyzed by HPLC using the following conditions: Solvent A was water containing 0.1% trifluoroacetic acid. Solvent B was acetonitrile/water (9:1 v/v) containing 0.086% trifluoroacetic acid. The gradient was 22−37% B linear in 15 min and 37−56% B linear from 15 to 45 min. The flow rate was 0.5 mL/min. Column was Zorbax 300SB C18 for peptides and proteins, 4.6 mm × 5 μm. Column temperature was 23 °C. The star marks an artifact peak. "N" indicates the elution position of the native αLA-IIIA. Two major fractions of scrambled three-disulfide isomers are marked as "a" and "c".

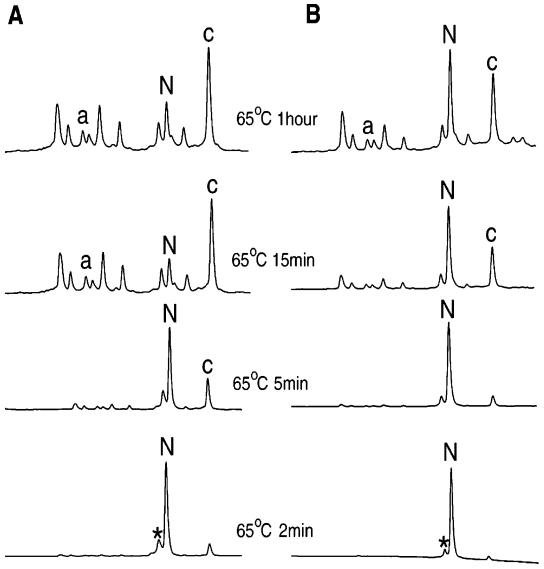

FIGURE 2: Thermal denaturation and unfolding of αLA-IIIA at 65 °C in the presence of thiol catalyst. Reactions were carried out both in the absence (A) and in the presence (B) of $CaCl_2$ (5 mM). Native αLA-IIIA (0.5 mg/mL) was denatured at 65 °C in Tris-HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.1 mM). Intermediates of denaturation were trapped at different time points with an equal volume of 4% aqueous trifluoroacetic acid and analyzed by HPLC using the conditions described in the legend of Figure 1. "N" indicates the elution position of the native αLA-IIIA. Two major fractions of scrambled three-disulfide isomers are marked as "a" and "c".

of αLA-IIIA is defined by the state of denatured αLA-IIIA and is structurally characterized by the composition (relative concentration) among the scrambled isomers.

Carboxymethylated αLA-IIIA was allowed to denature and unfold by shuffling its three native disulfides, transforming into a mixture of scrambled isomers, and reaching equilibrium after overnight incubation. For each denaturing condition, the experiments were performed both in the absence and in the presence of $CaCl_2$ (5 mM). To ensure that partial reduction of disulfide bonds plays an insignificant role in the process of denaturation, the entire population of denatured isomers from selected samples (6 M GdmCl and 8 M urea) were derivatized with vinylpyridine and analyzed by MALDI mass spectrometry. The data confirm that all intermediates appear in every HPLC profile shown are scrambled isoforms of αLA-IIIA. No reduction of any population was found (data not shown).

The structures of αLA-IIIA, denatured by increasing concentrations of GdmCl and analyzed by RP-HPLC, are presented in Figure 1. The results show that denaturation transformed native αLA-IIIA into a heterogeneous population of scrambled isomers. Out of a total of 14 possible disulfide isomers, 9 of them can be easily distinguished and identified. Urea has a similar effect on αLA-IIIA denaturation and unfolding, albeit with less potency (chromatogram not shown). In both cases, denatured αLA-IIIA consists of a steady predominant isomer (designated as X-αLA-IIIA-**c**) and a variable isomer the recovery of which is positively correlated to the concentration of denaturant (designated as X-αLA-IIIA-**a**) (Figure 1). The increased yield of X-αLA-IIIA-**a** is most remarkable at high concentration of GdmCl. The presence of $CaCl_2$ enhances stability of native αLA-IIIA against denaturation but has a minor bearing on the composition of denatured isomers.

Thermal Denaturation and Unfolding of αLA-IIIA. Thermal denaturation of native αLA-IIIA was carried out at 65 °C in the absence and presence of $CaCl_2$ (5 mM). Since prolonged incubation at high temperatures and alkaline pH (>9.0) may lead to the decomposition of disulfide bonds (*24*), thermal denaturation of αLA-IIIA was therefore performed in a time course manner for up to 60 min. The intermediate of unfolding was similarly quenched by acidification at selected time points and analyzed by HPLC.

Figure 2 displays the HPLC profiles of the scrambled isomers of αLA-IIIA denatured by heat in the absence and presence of $CaCl_2$. All nine different isoforms found by GdmHCl and urea denaturation can be distinguished after a 1 h incubation. The presence of $CaCl_2$ also strongly inhibits disulfide scrambling by thermal denaturation. Nevertheless, quantitative denaturation of αLA-IIIA cannot be achieved under these conditions, even in the absence of $CaCl_2$. Heat-denatured αLA-IIIA comprises one predominant isomer X-αLA-IIIA-**c** and a negligible concentration of X-αLA-IIIA-**a**, suggesting that heat is less effective in denaturing αLA-IIIA and that X-αLA-IIIA-**c** represents a partially unfolded structure of the native αLA-IIIA.

Denaturation Curves and Conformational Stability of αLA-IIIA. The denaturation curves of αLA-IIIA are determined by the fraction (%) of the native αLA-IIIA converted to the scrambled isomers X-αLA-IIIA under increasing

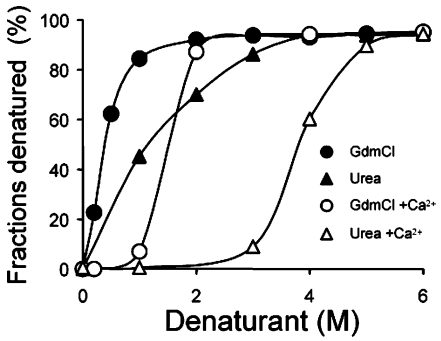

FIGURE 3: GdmCl and urea denaturation curves of αLA-IIIA. These curves were derived from HPLC data presented in Figure 1. Denaturation by GdmCl and urea were performed both in the absence and in the presence of $CaCl_2$ (5 mM). Fractions denatured indicate the fraction (%) of N-αLA-IIIA converted to scrambled isomers X-αLA-IIIA.

Table 1: Free Energy of GdmCl Induced Unfolding of Native αLA and αLA-IIIA[a]

proteins	$[GdmCl]_{1/2}$ (M)	m (kcal/(mol·M))	ΔG^{H_2O} (kcal/mol)	ΔG^{H_2O} (kcal/mol)[b]
αLA	1.1[c]	2.3	2.53	(3.66)
αLA (5 mM $CaCl_2$)	2.15[c]	2.1	4.52	(6.98)
αLA-IIIA	0.4	3.4	1.36	(1.26)
αLA-IIIA (5 mM $CaCl_2$)	1.4	2.4	3.36	(3.86)

[a] Calculated from $\Delta G_{app} = \Delta G^{H_2O} - m[GdmCl]$ at 23 °C in 0.1 M Tris-HCl, pH 8.4. $[GdmCl]_{1/2}$ is the midpoint of the GdmCl denaturation curves, m is the dependence of ΔG_{app} on GdmCl concentration. The error limit of ΔG^{H_2O} is ±0.4 kcal/mol. Midpoint of GdmCl denaturation is estimated from the denaturation curves given in Figure 4. [b] Values given in parentheses are free energy of unfolding of α-LA and α-LA-IIIA (identical to 2CM-3SS-BLA) analyzed by the "disulfide intact" denaturation and reported by Ikeguchi et al. (*19*). [c] Data reported by Chang and Li (*21*).

concentrations of a selected denaturant. Quantitative analysis of the relative yield of scrambled and the native isomers was based on the integration of HPLC peak areas. The urea and GdmCl denaturation curves of calcium-free and calcium-bound αLA-IIIA are shown in Figure 3. Concentrations of GdmCl needed to reach midpoint denaturation are estimated from Figure 3 and transformed into conformational free energy given in Table 1.

The conformational stability of αLA-IIIA obtained here is compared to the data of native αLA characterized by the same method of "*disulfide scrambling*" denaturation (columns 2−4 of Table 1) (*21*). Calcium is known to enhance the stability of αLA (*19*, *25*, *26*). It has a similar effect on αLA-IIIA. The increments of ΔG^{H_2O} caused by the addition of 5 mM $CaCl_2$ are exactly 2.0 kcal/mol each for both native αLA and αLA-IIIA. These data demonstrate that the stabilizing effect of calcium in the case of αLA-IIIA denaturation is as profound as that observed in the case of αLA, which strongly confirms that the calcium binding site of αLA-IIIA remains largely intact in the absence of the Cys^6-Cys^{120} disulfide bond. The difference in ΔG^{H_2O} between αLA and αLA-IIIA is 1.17 kcal/mol in the absence of calcium and 1.16 kcal/mol in the presence of 5 mM $CaCl_2$.

These results are further compared to the conformational free energy of αLA and αLA-IIIA (also known as 2CM-3SS−BLA) evaluated by the method of "disulfide intact" denaturation (*19*) (column 5 of Table 1). In these studies, the difference in ΔG^{H_2O} between αLA and αLA-IIIA is 2.4 kcal/mol in the absence of calcium and 3.1 kcal/mol in the presence of 5 mM $CaCl_2$. In addition, stability of αLA determined by the method of "disulfide intact" denaturation is invariably greater than that evaluated by the method of "disulfide scrambling" denaturation. The difference, $\Delta(\Delta G^{H_2O})$, of 1.13 kcal/mol (without calcium) and 2.46 kcal/mol (with calcium) reflects the conformational stability contributed by the *framework* of four native disulfide bonds, a phenomenon that has been elaborated in the case of phospholipase A_2 (*27*). In contrast, free energies of αLA-IIIA determined by both "disulfide intact" and "disulfide scrambling" denaturation techniques are roughly indistinguishable, a discrepancy that implies diminished reliance of the global stability of αLA-IIIA on its disulfide network.

*Disulfide Structures of X-αLA-IIIA-**a** and X-αLA-IIIA-**c***. Purified X-αLA-IIIA-**a** and X-αLA-IIIA-**c** were digested by thermolysin. Peptides were isolated by HPLC and characterized by Edman sequencing and MALDI mass spectrometry to identify the peptides that contain disulfide bonds. Peptide fractions that appear in both samples are those that contain noncysteine peptides and were sequenced only once. As peptide bonds between all adjacent cysteines can be cleaved by thermolysin, the interpretation of most data is rather straightforward. The results lead to the conclusion of the disulfide pairings of the two isomers of αLA-IIIA (Figure 4, left panel) (data are available upon request). X-αLA-IIIA-**c** was found to contain two scrambled disulfide bonds in the β-sheet region but still preserve the native disulfide bond $Cys^{28}-Cys^{111}$ in the α-helical domain. X-αLA-IIIA-**a**, which presents only under strong denaturing conditions, adopts the bead-form pattern with three disulfide bridges formed by consecutive cysteines.

*CD Spectra of X-αLA-IIIA-**a** and X-αLA-IIIA-**c***. Far-UV CD spectra for N-αLA, N-αLA-IIIA, X-αLA-IIIA-**c**, and X-αLA-IIIA-**a**, measured in the Tris-HCl buffer (20 mM, ph 7.4), are shown in Figure 5. The spectra of N-αLA-IIIA and N-αLA are very similar. Both display a double minimum at 208 and 222 nm, characteristic for α-helix-rich proteins, and a minimum also at approximately 216 nm, characteristic of the β-sheet-rich proteins. The difference of intensity is due to variation of sample concentration. These results are in agreement with previous studies performed with αLA variants (*19*, *20*). and confirm that the conformation of N-αLA-IIIA is essentially identical to that of N-αLA.

On the other hand, the CD spectra of X-αLA-IIIA-**c** and X-αLA-IIIA-**a** differ noticeably from that of N-αLA-IIIA. A significant loss of ellipticity is observed at the wavelength characteristic of β-sheet structure, a signal of substantial unfolding of both isomers at the β-sheet domain, consistent with their disulfide structures. At the same time, the minimum at 222 nm is also disappearing, but there are remains at a minimum close to the 208 nm region, at 205 nm for X-αLA-IIIA-**c** and 203 nm for X-αLA-IIIA-**a**. Since both scrambled isomers lack a clear single minimum at 200, which is the corresponding signal for the random coil structure, it is difficult to confirm the complete loss of secondary structure for them. Nonetheless, the data indicate the presence of a residual content of α-helix structure in X-αLA-IIIA-**c** and a closer to random coil structure of X-αLA-IIIA-**a**.

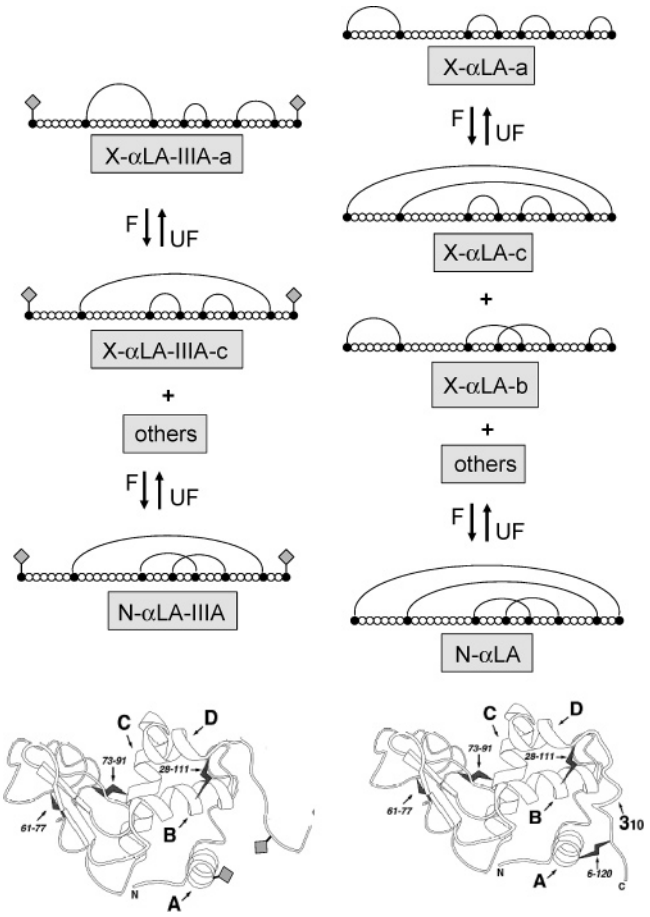

FIGURE 4: Major unfolding−refolding pathways of αLA and αLA-IIIA elucidated by the technique of disulfide scrambling. Free thiol groups of Cys^6 and Cys^{120} were blocked by carboxymethylation. Data supporting the pathways of αLA were documented in our previous studies (*21*, *22*).

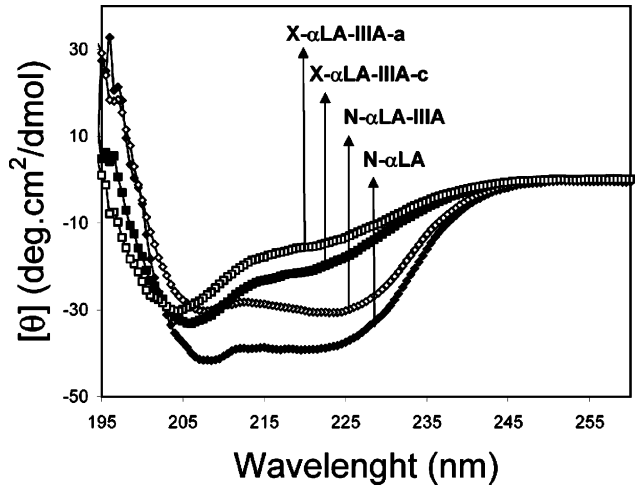

FIGURE 5: Far-UV CD spectra of (◆) N-αLA, (◇) N-αLA-IIIA, (■) X-αLA-IIIA-**c**, and (□) X-αLA-IIIA-**a**. Measurements were performed in the Tris-HCl buffer (20 mM, ph 7.4) at 23 °C.

Refolding of Unfolded Isomers of αLA-IIIA. Purified X-αLA-IIIA-**a** and X-αLA-IIIA-**c** were able to refold spontaneously to form the native αLA-IIIA in the Tris-HCl buffer (pH 8.4) containing a thiol catalyst (Figure 6). Folding can be achieved quantitatively with both isomers either in the absence or presence of $CaCl_2$. X-αLA-IIIA-**c** refolds to form N-αLA-IIIA with little accumulation of intermediates, implying that the major route of refolding involves direct

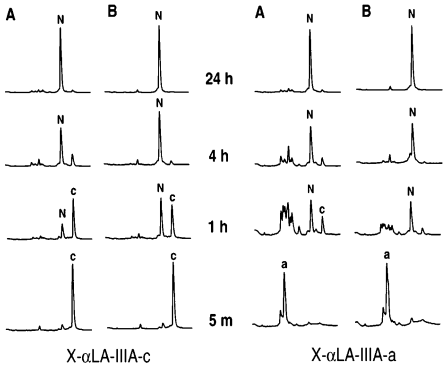

FIGURE 6: Refolding of X-αLA-IIIA-**c** and X-αLA-IIIA-**a** to form N-αLA-IIIA by disulfide scrambling. Refolding was performed at 23 °C in the Tris-HCl buffer (pH 8.4) containing 2-mercaptoethanol (0.1 mM). The reaction was carried out in the absence (A) and presence (B) of $CaCl_2$ (5 mM). The protein concentration was 0.5 mg/mL. Intermediates of refolding were trapped at different time points by sample acidification (4% trifluoroacetic acid) and analyzed by HPLC using the conditions described in the legend of Figure 1. "N" indicates the elution of N-αLA-IIIA.

isomerization of the two disulfide bonds within the *β*-sheet region. In contrast, X-αLA-IIIA-**a** appears to refold via diverse isomers, including X-αLA-IIIA-**c** as a major intermediate, to reach the native structure. These results further suggest that X-αLA-IIIA-**c** is a partially unfolded isomer of αLA-IIIA and one of the predominant intermediates for the unfolding of N-αLA-IIIA and refolding of X-αLA-IIIA-**a**. The presence of $CaCl_2$ (5 mM) increases the folding rate of X-αLA-IIIA-**a** and X-αLA-IIIA-**c** by about 3- and 2-fold, respectively. However, it has little influence on their refolding pathway.

A major pathway for the reversible unfolding and refolding of αLA-IIIA is depicted in Figure 4. In this scheme, X-αLA-IIIA-**c** represents among other diverse isomers the most prevalent unfolding/refolding intermediate and constitutes about 20−30% of the total intermediates identified. The pathway of αLA-IIIA is compared to those obtained from the studies of αLA (*21*, *22*), which is also shown in Figure 4.

DISCUSSION

Unfolding and Refolding Pathways of αLA-IIIA. αLA-IIIA is a three-disulfide kinetic trap present along the pathway of oxidative folding of reduced αLA. We have analyzed the unfolding and refolding pathways of αLA-IIIA using the method of "disulfide scrambling". To perform such analysis, free Cys^6 and Cys^{120} have to be chemically modified by thiol-specific reagent. Carboxymethylated αLA-IIIA is also known as 2CM-3SS-BLA or 3SSCOOH-αLA in earlier reports (*19*, *20*, *28*). The unfolding and refolding of 2CM-3SS-BLA have been investigated previously by the method of "disulfide-intact" denaturation using equilibrium and kinetic circular dichroism measurements (*19*). Our results of αLA-IIIA obtained here by the method of "disulfide-scrambling" confirm numerous properties observed with 2CM-3SS-BLA, which include the stabilizing effect of calcium and the relative stability between αLA and αLA-IIIA (Table 1). Specifically, a stable unfolding/refolding intermediate detected by stop-flow circular dichroism (*19*) correlates well with the predominant intermediate, X-αLA-IIIA-**c**, identified in our studies.

A major contribution of this presented study is the isolation of two chemically defined unfolding intermediates, X-αLA-IIIA-**c**, which predominates at mild denaturing conditions, and X-αLA-IIIA-**a**, which prevails under strong denaturing conditions. Their disulfide structures, CD spectra, and manners of refolding are all consistent with the notion that X-αLA-IIIA-**c** and X-αLA-IIIA-**a** represent a partially and an extensively unfolded isomer of native αLA-IIIA, respectively.

X-αLA-IIIA-**c** comprises two scrambled disulfide bonds at the *β*-sheet domain but still preserves the native disulfide bond (Cys^{28}−Cys^{111}) within its α-helical region (Figure 4). This structure is consistent with its CD spectrum, which exhibits residual α-helical content and a significant loss of *β*-sheet structure (Figure 5). The disulfide structure of X-αLA-IIIA-**c** is also compatible with its pathway of refolding to form N-αLA-IIIA, which engages direct shuffling of the two non-native disulfide bonds within the *β*-sheet domain (Figure 6).

On the other hand, X-αLA-IIIA-**a** adopts "bead-form" disulfide pairing (Figure 4). This disulfide pattern represents an extensively unfolded state and is common among disulfide isomers that predominate under strong denaturing conditions (*23*, *29*, *30*). The CD spectrum also indicates that the structure of X-αLA-IIIA-**a** is close to a random coil (Figure 5). Most revealing is the pathway of refolding of X-αLA-IIIA-**a**, which undergoes heterogeneous isomers including X-αLA-IIIA-**c** to reach N-αLA-IIIA (Figure 6).

The major unfolding and refolding pathways of αLA-IIIA are outlined in Figure 4. Despite the absence of the Cys^{6}−Cys^{120} disulfide bond, they bear a striking resemblance to the unfolding/refolding pathways of αLA elucidated by the same method (*21*, *22*). These similarities involve (a) the extent of heterogeneity of unfolding/refolding intermediates (more than 50% of potential scrambled isomers are found as intermediates in both αLA and αLA-IIIA) and (b) the presence of partially unfolded/refolded isomers that exhibit properties of molten globule. X-αLA-IIIA-**c** and X-αLA-**c** are two corresponding predominant intermediates with their *β*-sheet domain selectively unfolded. These two isomers together with X-αLA-**b** (Figure 4) in our opinion constitute the major structure of molten globule of αLA-IIIA and αLA observed by many different laboratories.

Molten Globule of αLA and Unfolding Intermediates of αLA-IIIA. Molten globules are generally defined as partially structured proteins with a high degree of secondary structure but with a loosened tertiary fold (*3*, *6*). They have been proposed to be common intermediates in protein folding. However, an explicit description of a molten globule remains elusive and controversial. It is likely that the structure of a molten globule consists of a collection of isomers with distinctive nativelike topology at different parts of the protein. The structure of αLA induced at low pH (often referred to as A-state) is one of the first and best-characterized molten globules (*2*, *8*). αLA represents an ideal model for demonstrating the properties of molten globule partly because its two distinct α-helical and *β*-sheet domains can be selectively unfolded under different denaturing conditions (*21*). Mounting effort using αLA variants has been undertaken by different laboratories to elucidate the structure of molten globules and elements that stabilize them (*31*−*33*).

Redfield et al. (*13*) demonstrated that αLA may form a compact molten globule in the absence of all four disulfide bonds. Luo and Baldwin (*14*) showed that the Cys^{28}−Cys^{111} disulfide bond constrains the αLA molten globule and weakens its cooperativity of folding. Horng et al. (*20*) revealed that the A-state molten globule of αLA may not be a rigorous mimic of the folding intermediate populated at neutral pH. Wu and Kim (*34*) and Song et al. (*35*) showed that a subset of hydrophobic amino acid residues within the helical domain is most important for formation of nativelike topology of αLA molten globule.

Most significantly, Kim and co-workers (*34*, *36*, *37*) have shown that the nativelike topology of αLA molten globule does not encompass the entire polypeptide chain. Molten globule of αLA comprises a nativelike, structured α-helical domain and a largely disordered *β*-sheet domain, a structure that is conspicuously identical to X-αLA-**c** and similar to X-αLA-IIIA-**c** (Figure 4). Further studies using a variant, αLA(*β*), which contains only two native disulfide bonds within the *β*-sheet domain, suggest that organization of the *β*-sheet domain represents a locking step in the folding of αLA from the molten globule to the native state (*15*). The structure of αLA(*β*) is compatible with X-αLA-**b** (Figure 4), a major unfolding/refolding intermediate identified together with X-αLA-**c** by the method of "disulfide scrambling" (*21*).

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