The unfolding mechanism and the disulfide structures of denatured lysozyme

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Abstract The mechanism of denaturation and unfolding of lysozyme has been characterized here using the method of disulfide scrambling. Under denaturing conditions (urea, guanidinium hydrochloride (GdmCl), guanidinium thiocyanate (GdmSCN), or elevated temperature) and in the presence of thiol initiator, lysozyme denatures by shuffling its four native disulfide bonds and converts to a mixture of fully oxidized scrambled isomers. To denature 50% of the native lysozyme requires 1.1 M of GdmSCN, 2.8 M of GdmCl and 7.4 M of urea, respectively. High temperature (75°C) denatures the native lysozyme quantitatively within 20 min. Analysis by reversed-phase high-performance liquid chromatography reveals that urea and GdmCl denatured lysozyme comprise a single predominant disulfide isomer, designated as X-lysozyme-a, regardless of the concentration of the denaturant. X-Lysozyme-a was shown to adopt the *beads-form* structure with its four disulfide bonds formed by four consecutive pairs of cysteines (Cys^{6}–Cys^{30}, Cys^{64}–Cys^{76}, Cys^{80}–Cys^{94}, Cys^{115}–Cys^{127}). The conspicuous absence of partially structured unfolding intermediates of lysozyme contrasts to that found in the case of α-lactalbumin and accounts for the widely observed two-stage mechanism of lysozyme unfolding.

Key words: Lysozyme; α-Lactalbumin; Urea; Guanidinium hydrochloride; Guanidinium thiocyanate; Denaturation; Thermal denaturation; Unfolding; Denaturation curve; Unfolding curve; Scrambled lysozyme

1. Introduction

Lysozyme represents one of the most extensively investigated models for understanding the mechanism of protein stability, folding and denaturation [1–14]. Like most proteins, denatured lysozyme may comprise a mixture of conformational isomers that exist in a state of thermodynamic equilibrium [15,16]. So far, characterization of denatured lysozyme has been largely achieved by measuring the average properties of these collective isomers using a wide range of spectroscopic and physiochemical methods, including circular dichroism (CD) [1,5,10] and nuclear magnetic resonance (NMR) [4,7,14]. Lysozyme is an attractive model in part due to its unique structure–function relationship to another widely investigated protein, α-lactalbumin [1,17–32]. Comparative analysis of their folding–unfolding mechanisms has been the subject of intensive investigation [1]. Lysozyme and α-lactalbumin share structural homology of their primary sequences (∼40%), disulfide patterns and three-dimensional conformations. Despite that, these two proteins exhibit distinctive biological functions and physicochemical properties. While lysozyme is a catalytic enzyme, α-lactalbumin is a calcium binding protein required for synthesis of lactose. The most important difference of structural properties between lysozyme and α-lactalbumin is revealed by the mechanism of their denaturation. In contrast to lysozyme, which undergoes the two-state thermal denaturation [1,12], α-lactalbumin follows a three-state model and forms an intermediate 'molten globule' state [18–21]. The structure of α-lactalbumin molten globule is characterized by a high degree of native-like secondary structure and a fluctuated tertiary fold [18,19]. It consists of a structured α-helical domain and an unstructured, disordered β-sheet region [28,29]. The absence of molten globule for the homologous lysozyme has long been baffling and is believed to be due to its structural variation at the β-sheet domain (amino acid residues 76–102).

Further understanding of the molecular mechanism of two-stage unfolding of lysozyme will require structural analysis of denatured lysozyme and fractionation of diverse populations of conformational isomers that constitute the denatured lysozyme. In this report, the mechanism of denaturation and unfolding of lysozyme has been analyzed by the technique of disulfide scrambling [33,34]. In the presence of denaturant and a thiol initiator, the native lysozyme denatures by shuffling its native disulfide bonds and converts to a mixture of fully oxidized scrambled isomers that are trapped by non-native disulfide bonds. Lysozyme contains four disulfide bonds and may adopt 104 possible scrambled isomers. The technique of disulfide scrambling presents two major advantages for characterization of the denaturation of lysozyme. (1) Denatured isomers of lysozyme are stable under acidic conditions. They can be fractionated and purified by liquid chromatography, and structurally characterized. This permits a detailed description of the structure of denatured lysozyme that is not affordable with conventional spectroscopic methods. (2) Denatured scrambled isomers of lysozyme contain different sizes of disulfide loops and adopt a varied degree of unfolding. Compositional analysis of scrambled isomers allows evaluation of the extent of unfolding of the denatured lysozyme.

Our specific aims of this study are: (1) to analyze the conformational stability of lysozyme using the technique of disul-

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Abbreviations: X-Lysozyme, scrambled lysozyme; GdmCl, guanidinium hydrochloride; GdmSCN, guanidinium thiocyanate

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fide scrambling, (2) to characterize the structure and heterogeneity of denatured lysozyme, and (3) to elucidate the unfolding pathway of lysozyme denatured under increasing concentrations of selected denaturants.

2. Materials and methods

2.1. Materials

Chicken egg lysozyme (L-6876) was obtained from Sigma. The protein was further purified by high-performance liquid chromatography (HPLC) and was shown to have a purity of more than 97%. Guanidinium hydrochloride (GdmCl), guanidinium thiocyanate (GdmSCN), urea, and 2-mercaptoethanol were also purchased from Sigma and had a purity grade of greater than 99%.

2.2. Denaturation and unfolding of the native lysozyme

The native lysozyme (0.5 mg/ml) was dissolved in the Tris–HCl buffer (0.1 M, pH 8.4) containing 2-mercaptoethanol (0.1–0.2 mM) and selected concentrations of denaturants (urea, GdmCl, GdmSCN). Denaturation and unfolding were typically performed at room temperature (23°C) for 20 h to ensure that conversion of the native lysozyme to scrambled isomers has reached the state of equilibrium. In the case of thermal denaturation, there are inherent difficulties of permitting the process of denaturation to reach a state of equilibrium, due to the destruction of disulfide bonds during prolonged sample heating. Thermal denaturation of lysozyme was therefore preformed in a kinetic fashion. The sample (in the presence of 0.1 mM 2-mercaptoethanol) was subjected to elevated temperatures (55–75°C) for a time period of up to 60 min. To monitor the kinetics and intermediates of unfolding, aliquots of the sample were removed in a time-course manner, quenched with an equal volume of 4% aqueous trifluoroacetic acid and analyzed by reversed-phase HPLC. The denatured sample was subsequently acidified with 4% trifluoroacetic acid and stored at −20°C.

2.3. Denaturation is distinguished from unfolding

The denatured lysozyme may adopt a varied extent of unfolding. Denaturation and unfolding are therefore two distinctive terms. Using the method of disulfide scrambling [33,34], it is feasible to observe and follow simultaneously the process of denaturation and unfolding of lysozyme. The extent of denaturation of lysozyme is defined by the conversion of the structure that contains four native disulfide bonds to scrambled isomers (non-native structures). There are 104 scrambled isomers of denatured lysozyme versus one isomer of the native lysozyme. Unfolding of lysozyme is defined by the state of denatured lysozyme and is structurally characterized by the composition (relative concentration) among the 104 possible scrambled isomers.

2.4. Plotting of the denaturation curves of lysozyme

The denaturation curve of lysozyme was determined by the fraction (%) of the native lysozyme converted to the scrambled isomers. Quantitative analysis of the relative yield of scrambled and the native isomer was based on the integration of HPLC peak areas.

2.5. Plotting of the unfolding curves of lysozyme

The unfolding curve of lysozyme was determined by the composition of denatured (scrambled) lysozyme along the pathway of unfolding. Calculation of the yield of scrambled isomers was based on the peak area integration. Due to the complexity of minor isomers, the data have a standard deviation of ± 5%.

2.6. Structural analysis of scrambled isomers of lysozyme

Fractions of scrambled lysozyme (~10 μg) were isolated and treated with 1 μg of thermolysin 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 amino acid sequencing and mass spectrometry in order to identify the disulfide-containing peptides.

2.7. Amino acid sequencing and mass spectrometry

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 peptides were determined by a matrix-assisted laser desorption ionization (MALDI)-time of flight mass spectrometer (Perkin-Elmer Voyager-DE STR).

2.8. Nomenclature of scrambled isomers of lysozyme

Scrambled species of lysozyme are designated by the following formula: X-lysozyme-(species assigned on HPLC), where X stands for scrambled. For instance, X-lysozyme-a represents species 'a' of scrambled lysozyme. Similar formula of nomenclature are applied to scrambled isomers of α-lactalbumin.

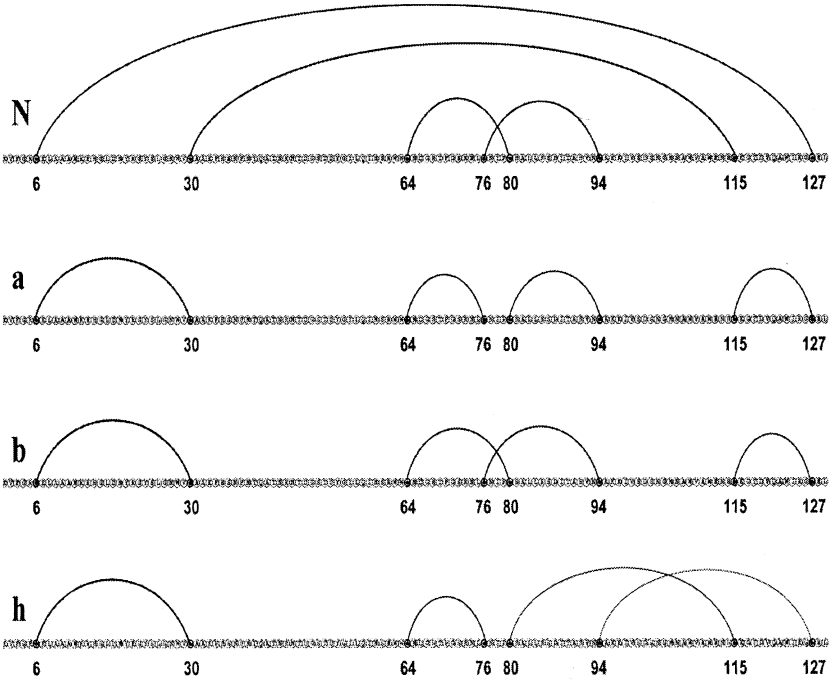

Fig. 1. Disulfide structures of the native lysozyme and three isomers of scrambled lysozyme (a, b and h). Their structures were derived from the Edman sequencing and mass analysis of disulfide-containing peptides of thermolysin-digested samples (data not shown).

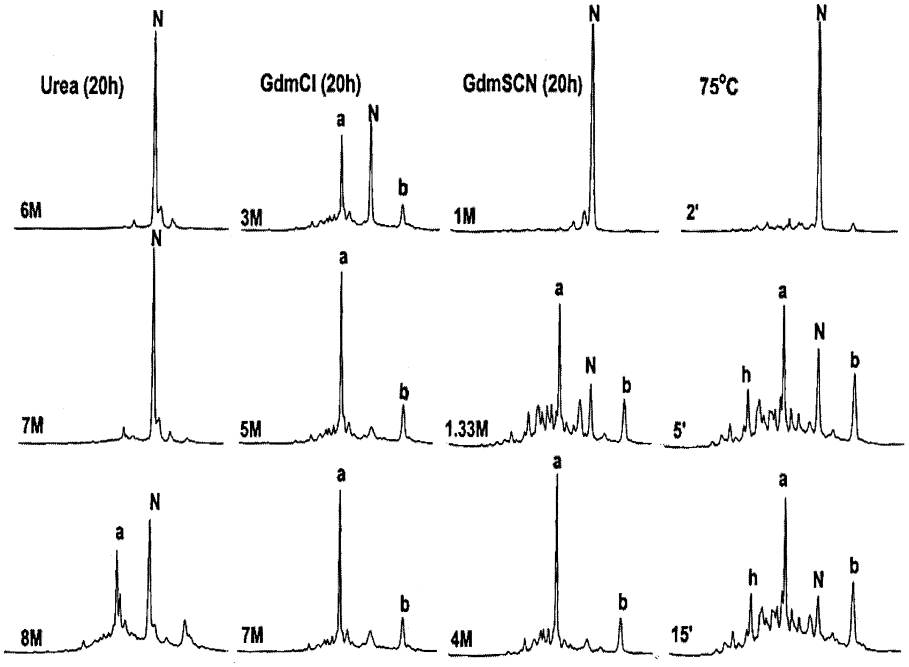

Fig. 2. Scrambled isomers of lysozyme generated by denaturation by elevated temperature (75°C) and by different concentrations of urea, GdmCl and GdmSCN. Chemical denaturation was carried out at 23°C for 20 h in the Tris–HCl buffer (pH 8.4) containing 2-mercaptoethanol (0.1 mM) and selected denaturant. Thermal denaturation was performed in a time-course manner in the same buffer containing 2-mercaptoethanol (0.1 mM). Denatured samples were acidified with 4% trifluoroacetic acid and analyzed by reversed-phase HPLC using the following conditions. Solvent A for HPLC was water containing 0.1% trifluoroacetic acid. Solvent B was acetonitrile/water (9:1, by volume) containing 0.086% trifluoroacetic acid. The gradient was 20% B to 34% B in 15 min, 34% B to 53% B from 15 to 60 min. The flow rate was 0.5 ml/min. The column was Zorbax 300SB-C18 for peptides and proteins, 4.6 mm, 5 μm. The column temperature was 23°C. The concentration of denaturant and three predominant isomers (a, b and h) of denatured lysozyme are marked.

3. Results

3.1. Disulfide structures of the predominant isomers of denatured lysozyme

Lysozyme contains four disulfide bonds and may adopt 104 possible non-native scrambled isomers. The structure of denatured lysozyme was found to consist of at least 25 fractions of both predominant and minor scrambled isomers. Their relative concentrations vary under different denaturing conditions (see the following sections). Among them, three major fractions of scrambled lysozyme were isolated and structurally characterized. They are X-lysozyme-a, X-lysozyme-b and X-lysozyme-h (Fig. 1). They were digested by thermolysin. Peptides were isolated by HPLC and characterized by Edman sequencing and MALDI mass spectrometry in order to identify the peptides that contain disulfides (data available upon request). Their disulfide structures are presented in Fig. 2. X-Lysozyme-a represents the most predominant species under all conditions investigated. The disulfide bonds of X-lysozyme-a are formed by four pairs of consecutive cysteines (Cys^{6}–Cys^{30}, Cys^{64}–Cys^{76}, Cys^{80}–Cys^{94}, Cys^{115}–Cys^{127}). It contains, among the 104 possible isomers, the smallest sizes of combined disulfide loops and presumably corresponds to the most extensively unfolded structures of the denatured lysozyme.

3.2. Structures of lysozyme denatured by urea, GdmCl and GdmSCN

Thermodynamic denaturation of lysozyme was performed in the presence of 2-mercaptoethanol (0.1 mM) and increasing concentrations of urea, GdmCl and GdmSCN. All denaturation experiments were performed for 20 h to ensure that the reaction reaches the state of equilibrium. Chromatograms of denatured lysozyme are given in Fig. 2. The disulfide structure of GdmSCN denatured lysozyme appears to be more heterogeneous than that denatured by urea and GdmCl. However, all denatured samples consist of two major scrambled isomers,

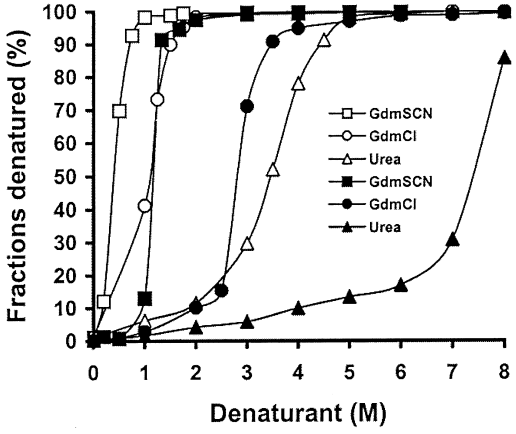

Fig. 3. Denaturation curves of lysozyme (by urea, GdmCl and GdmSCN). These curves are derived from analysis of the relative recoveries of the native and scrambled lysozyme generated under increasing concentrations of GdmSCN (■), GdmCl (●), and urea (▲) (chromatograms shown in Fig. 2). Denaturation was performed at 23°C for 20 h to allow the reaction to reach the state of equilibrium. Fractions denatured indicate the fraction (%) of native lysozyme converted to scrambled lysozyme. The corresponding denaturation curves of α-lactalbumin (calcium-depleted) obtained by the same method under increasing concentrations of GdmSCN (□), GdmCl (○), and urea (△) are also presented [34].

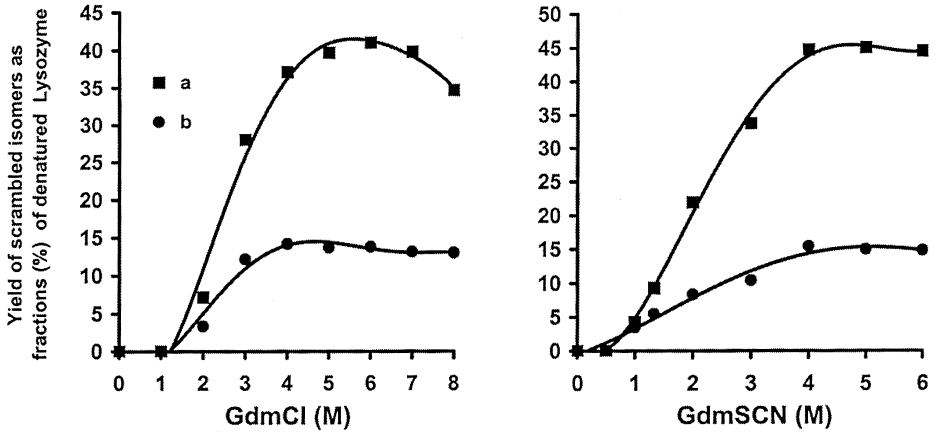

Fig. 4. Unfolding curves of lysozyme (by GdmCl and GdmSCN). These curves were derived from compositional analysis of lysozyme denatured under increasing concentrations of GdmCl and GdmSCN. The recoveries of two predominant isomers, X-lysozyme-a (■) and X-lysozyme-b (●), as fractions of the total denatured protein (scrambled isomers) were used to construct these curves.

X-lysozyme-a and X-lysozyme-b (their disulfide structures are shown in Fig. 1).

The denaturation curves of lysozyme (Fig. 3) reveal that GdmSCN is about 2.5-fold and 7-fold more potent than GdmCl and urea, respectively (Table 1). Such a comparative potency of urea, GdmCl and GdmSCN has been similarly observed in the case of denaturation of α-lactalbumin [34], in which GdmSCN was shown to be 2.7-fold and 8-fold more potent than GdmCl and urea (Table 1). The unfolding curves (Fig. 4) further display the structures of denatured lysozyme evolved under increasing concentrations of two selected denaturants (GdmCl and GdmSCN). Characteristically, the progressive unfolding of denatured lysozyme is accompanied by an increasing yield of X-lysozyme-a and X-lysozyme-b, two isomers that apparently represent the most unfolded structures among the 104 possible scrambled isomers of lysozyme. For instance, the recovery of X-lysozyme-a, as a fraction of the total scrambled lysozyme, increases by 9-fold, from 5% to 45%, as the concentration of GdmSCN raises from 1 M to 6 M (Fig. 4). A similar phenomenon was observed with the GdmCl denaturation of lysozyme.

3.3. Thermal denaturation of lysozyme

The native lysozyme was denatured at 55°C, 65°C and 75°C. Intermediates were trapped by acidification at different time points and analyzed by HPLC (Fig. 2, right column). The extent of denaturation was measured by the fraction of native protein converted to scrambled isomers (Fig. 5). The native lysozyme was shown to be completely stable at 55°C for up to 60 min. At a higher temperature (65°C), about 55% of the native protein becomes denatured under otherwise similar conditions. The initial rate of denaturation of lysozyme increases by nearly 14-fold as the temperature rises from 65°C to 75°C. The native lysozyme, as determined by the rate of thermal denaturation at 65°C, is approximately 7-fold more stable than calcium-depleted α-lactalbumin [34] (Fig. 5).

The structure of heat-denatured lysozyme is considerably more heterogeneous than that denatured by GdmCl and com-

Table 1
The concentration of denaturant required to denature 50% of the protein[a]

Proteins	Size of protein (amino acids)	No. of disulfides	GdmSCN (M)	GdmCl (M)	Urea (M)
α-Lactalbumin[c] (calcium-depleted)	122	4	0.4	1.1	3.4
PCI[d]	39	3	0.7	1.45	>8[b]
Ribonuclease A[e]	124	4	0.75	2.25	5.75
TAP[f]	122	3	1.0	4.2	4.0
Lysozyme	129	4	1.1	2.8	7.4
IGF-1[g]	70	3	1.5	3.2	5.5
Hirudin[h]	49	3	2	5	>8[b]
Epidermal growth factor (EGF)[i]	53	3	2.1	3.9	>8[b]
BPTI[j]	58	3	3.25	7.5	>8[b]

[a]Determined by the method of disulfide scrambling. The extent of denaturation is determined by the fraction of native protein converted to scrambled isomers. The data should be allowed a standard deviation of ±5%.
[b]At 8 M urea, 45% of PCI, 16% of hirudin, 14% of EGF and less than 2% of BPTI were denatured.
[c][34].
[d][39].
[e][41].
[f][33].
[g][36].
[h][38].
[i]Chang and Li, unpublished data.
[j][37].

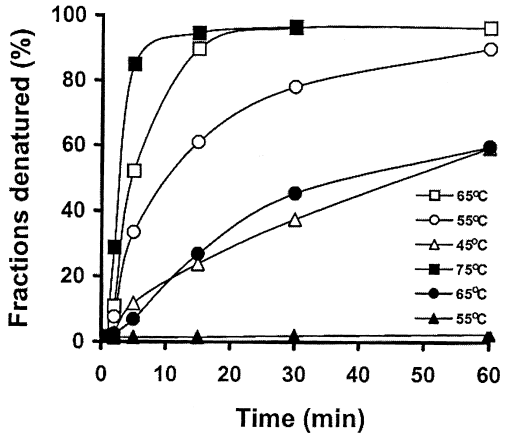

Fig. 5. Denaturation curves of lysozyme generated by thermal denaturation. Denaturation was performed at 75°C (■), 65°C (●), and 55°C (▲) for up to 60 min. Fractions denatured indicate the fraction (%) of native lysozyme converted to scrambled lysozyme. The corresponding denaturation curves of calcium-depleted α-lactalbumin obtained by the same method at 65°C (□), 55°C (○), and 45°C (△) are also presented [34].

prises many of the isomers found within the structure denatured by GdmSCN (Fig. 2). Among them, the three well populated species, X-lysozyme-a, X-lysozyme-b and X-lysozyme-h, account for about 40% of the total denatured structure of lysozyme.

4. Discussion

4.1. The conformational stability of lysozyme

The results obtained here allowed us to compare the conformational stability of lysozyme with several disulfide proteins using the same chemical marker (disulfide scrambling). Their relative stability is presented in Table 1. These results demonstrate that GdmSCN is typically 2–3-fold more potent than GdmCl, which in turn is an additional 2–3-fold more effective than urea [35]. However, it is important to mention that ranking of protein stability is also determined by the nature of denaturant. For instance, based on the urea denaturation curves, lysozyme is about 1.4-fold more stable than insulin-like growth factor (IGF-1) [36]. This relative stability is precisely reversed when comparison is based on GdmSCN denaturation curves (Table 1). Among those listed in Table 1, the stability of bovine pancreatic trypsin inhibitor (BPTI) is most remarkable. BPTI represents the only case that is totally resistant to denaturation by 6 M GdmCl or 8 M urea [37].

Lysozyme is more stable than calcium-depleted α-lactalbumin, a structurally homologous protein. Estimation from the data of chemical denaturants (urea, GdmCl and GdmSCN) indicates that lysozyme is about 2.5-fold more stable than α-lactalbumin. This conclusion is based on the relative concentration of denaturant required to achieve 50% denaturation of the protein thermodynamically (Table 1). In a separate kinetic analysis of thermal denaturation (at 65°C), lysozyme was shown to be 7-fold more stable than α-lactalbumin (Fig. 5).

4.2. The unfolding mechanism of lysozyme differs from that of α-lactalbumin, in which partially structured intermediates observed along the unfolding pathway of α-lactalbumin are conspicuously absent in the case of lysozyme

The unfolding curves of lysozyme are derived from the structural analysis of denatured lysozyme (composition of its scrambled species) under increasing concentrations of selected denaturants. Comparison of the data of lysozyme with those obtained from α-lactalbumin [34] reveals that both proteins do share a common property of unfolding of their denatured structures. This is characterized by a progressive expansion and relaxation of the protein conformation toward the shape of linear structure. Among the 104 possible scrambled isomers of lysozyme, X-lysozyme-a (the *beads-form*, see Fig. 2) contains the smallest disulfide loops and represents the most extensively unfolded structure. The yield of X-lysozyme-a is directly proportional to the strength of the denaturing condition. This phenomenon is most evident with samples denatured with GdmCl and GdmSCN. As the concentration of GdmSCN increases from 1 M to 6 M, the recovery of X-lysozyme-a as a fraction of the total denatured lysozyme grows from 5% to 45% (Fig. 4). In the case of α-lactalbumin, the yield of the *beads-form* isomer rises from 5% to 27% under similar conditions [34]. Lysozyme and α-lactalbumin are not unique cases. The predominance of *beads-form* isomer under strong denaturing conditions has also been observed with the unfolding behaviors of tick anticoagulant peptide (TAP) [33], IGF-1 [36], hirudin [38], potato carboxypeptidase inhibitor (PCI) [39] and BPTI [37]. For example, in the presence of 6 M GdmSCN, more than 63% of the denatured TAP and 55% of denatured BPTI were found to be the *beads-form* isomer. In the case of IGF-1, the yield of *beads-form* isomer also rises from 5 to 30% as the concentration of GdmSCN increases from 1 M to 6 M.

What distinguishes the unfolding pathway of lysozyme from that of α-lactalbumin is the absence of intermediate. Along the unfolding pathway of α-lactalbumin [34], a partially structured intermediate (designated as X-α-lactalbumin-c) was found to populate under mild denaturing conditions. At low concentrations of GdmSCN (0.75 M), X-α-lactalbumin-c constitutes approximately 10% of the total denatured α-lactalbumin. The prevalence of X-α-lactalbumin-c as an unfolding intermediate is most obvious with thermal denaturation in which X-α-lactalbumin-c accounts for more than 40% of the denatured α-lactalbumin during the early stage of kinetic denaturation [34]. X-α-Lactalbumin-c contains two native disulfide bonds (Cys^{6}–Cys^{120} and Cys^{28}–Cys^{111}) within the α-helical domain and two non-native disulfide bonds (Cys^{61}–Cys^{73} and Cys^{77}–Cys^{91}) within the β-sheet (calcium binding) domain of α-lactalbumin (Fig. 2). X-α-Lactalbumin-c also retains a substantial content of α-helical structure as measured by its far UV CD spectrum. [40]. The structural properties of X-α-lactalbumin-c bear a close resemblance to that of the well characterized molten globule of α-lactalbumin [18–32]. NMR analysis showed that the most persistent structure in the α-lactalbumin molten globule is localized at the α-helical domain [31]. Kim and colleagues have demonstrated that the molten globule properties of α-lactalbumin are mainly confined to one of its two domains. The α-helical domain forms a helical structure with a native-like tertiary fold, whereas the β-sheet domain is essentially disordered [28,29].

By contrast, we have not detected any significant concentration of the counterpart of X-α-lactalbumin-c along the unfolding pathway of denatured lysozyme, despite the structural homology between lysozyme and α-lactalbumin. This yet to be identified isomer of lysozyme (designated as X-lysozyme-c) should adopt the disulfide pairing of Cys^{6}–Cys^{127}, Cys^{64}–

Cys^{76}, Cys^{80}–Cys^{94}, Cys^{30}–Cys^{115}. The conspicuous absence of corresponding X-lysozyme-c as an unfolding intermediate of lysozyme represents a major hallmark of structural difference between lysozyme and α-lactalbumin. However, these data are fully consistent with documented observations of the differential folding behavior of lysozyme and α-lactalbumin. It accounts for the two-state model of lysozyme denaturation versus the three-state model of α-lactalbumin denaturation [1,12,18–21].

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