Analysis of the Early Stage of the Folding Process of Reduced Lysozyme Using All Lysozyme Variants Containing a Pair of Cysteines†

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ABSTRACT: Twenty-eight hen lysozyme variants that contained a pair of cysteines were constructed to examine the formation of the individual native and nonnative disulfide bonds. We analyzed the extent of the formation of a disulfide bond in each lysozyme variant using a redox buffer (pH 8) containing 1.0 mM reduced and 0.1 mM oxidized glutathione in the absence or presence of 6 M guanidine hydrochloride. In the presence of 6 M guanidine hydrochloride, the extent of the formation of the disulfide bond in each lysozyme variant was proportional to the distance between cysteine residues, indicating that reduced hen lysozyme under a highly denaturing condition adopted a randomly coiled structure. In aqueous solution, the formations of all disulfide bonds occurred much more easily than under a denatured condition. This finding indicated that reduced lysozyme had a somewhat compact structure. Moreover, the scattering data for the extents of the formation of the disulfide bonds among all lysozyme variants were observed. These results suggested that the nonrandom folding occurred in the early stage of the folding of reduced lysozyme, which should provide new insight into the early-stage events in the folding process of reduced lysozyme.

An improved understanding of protein folding is one of the most important goals in the field of post-genome-period life science, both because such understanding is indispensable to the de novo design of proteins (*1*, *2*) and because misfolding of proteins is associated with a wide range of diseases (*3*). Numerous experimental and theoretical advances have already led to considerable progress in our understanding of protein folding. However, the underlying factors that control the process of protein folding remain unknown. One of the most effective approaches for elucidating the factors involved in protein folding is to identify the folding intermediates (*4*). That is, we can determine the interactions in the folding process of a protein by elucidating the structure of the folding intermediates. Generally, the folding intermediates are difficult to study because they are transient and often present only at low concentrations. However, it is possible to trap such intermediates in the course of protein folding by analyzing the disulfide bond formations. The folding processes of a few proteins [e.g., bovine pancreatic trypsin inhibitor (*5*, *6*), ribonuclease T1 (*7*, *8*), ribonuclease A (*9*, *10*, *11*), and α-lactalbumin (*12*, *13*)] have been analyzed in detail as an indicator of the passage of folding by the formation of disulfide bonds.

Hen egg-white lysozyme is one of the proteins of which the folding processes from the reduced form have been investigated. More than 20 years ago, Anderson and Wetlaufer (*14*) suggested that the two disulfide bonds involving Cys64−Cys80 and Cys76−Cys94 formed earlier than those involving Cys6−Cys127 and Cys30−Cys115 in the folding of reduced hen lysozyme. This finding indicated that there was an order in the formation of disulfide bonds in the early stage of the folding of reduced lysozyme. Recently, van den Berg et al. (*15*) performed a detailed biophysical study of the folding of reduced lysozyme and provided new insight into the underlying determinants of the folding reaction, including the role of the disulfide bonds. They demonstrated that the folding process from the reduced form was heterogeneous and complex before the formation of the folding intermediate with three disulfide bonds. Namely, they suggested that no prominent disulfide bonds were formed in the early stage of the folding of reduced lysozyme. Therefore, the events in the early stage of the folding process of reduced hen lysozyme are now controversial.

Tachibana (*16*) constructed four species of hen lysozyme variants, each containing one intact disulfide bond, and evaluated how the individual disulfide bonds were formed. Their method can be used to clarify the interactions involved in the formation of disulfide bonds. However, since the native disulfide bond does not always form in the early stage of folding of reduced lysozyme, we should also pay attention to nonnative disulfide bonds. Therefore, to elucidate whether prominent formation of some disulfide bonds occurs in the early stage of the folding of reduced lysozyme, we prepared all possible species of hen lysozyme variants containing a single pair of cysteines (4 native species and 24 nonnative species) and evaluated the extent of individual disulfide bond formation. The results indicated that reduced hen lysozyme

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had a somewhat compact structure and that the nonrandom folding occurred in the early stage of the folding of reduced lysozyme.

MATERIALS AND METHODS

Materials. CM-Toyopearl 650 M and Cosmosil C18 were purchased from Tosoh Co., Ltd. (Tokyo) and Nacalai Tesque (Kyoto, Japan), respectively. The μ-BONDASPHERE C4 column (150 mm × 3.9 mm) was obtained from Waters Associates (Milford, MA). Oxidized and reduced glutathione were purchased from Nacalai Tesque (Kyoto). Guanidine hydrochloride was purchased from Kanto Chemical Co., Inc. (Tokyo). 3-Trimethylammoniopropylmethanethiosulfonate Bromide (TAPS) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of the highest quality commercially available.

Construction of a Lysozyme Variant Containing a Pair of Cysteines. Site-directed mutagenesis was performed by PCR according to the method described in our previous work (*17*). Namely, to improve physicochemical properties of protein, we expressed proteins as Ser^{-1} proteins instead of Met^{-1} proteins (*17*). The genes for lysozyme variants containing a pair of cysteines were constructed by using synthetic oligonucleotides that contained the codons for alanine residues in place of the respective cysteine residues. For example, the gene for the Cys64−Cys80 lysozyme variant was constructed by the replacements Cys6→Ala, Cys30→Ala, Cys76→Ala, Cys94→Ala, Cys115→Ala, and Cys127→Ala, resulting in a gene that retained only Cys64 and Cys80. *Nde*I and *Bam*HI restriction sites were introduced at the 5′- and 3′-end of the lysozyme variant gene, respectively. The digested fragment (*Nde*I/*Bam*HI) was inserted between the *Nde*I and *Bam*HI sites of the pET22b(+) vector (Novagen, Madison, WI) just downstream of the T7 promoter. The nucleotide sequencing was carried out by the dideoxy chain termination method.

Expression of a Lysozyme Variant Containing a Pair of Cysteines in Escherichia coli. An expression plasmid containing a gene for a lysozyme variant that contained a pair of cysteines was introduced into *E. coli* BL21(DE3) cells (Novagen). The resulting transformant was grown at 37 °C in LB medium. When the absorbance of the culture medium at 660 nm reached 0.8, isopropyl-β-D(−)-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM, and the transformant expressing a lysozyme variant containing a pair of cysteines was cultured for 4 h.

Purification of a Lysozyme Variant Containing a Pair of Cysteines. Purification of a lysozyme variant containing a pair of cysteines was carried out according to the method described in our previous work (*17*) with a slight modification. Namely, cells were harvested by centrifugation for 15 min at 8500 rpm. Pellets were suspended in 30 mL of 50 mM MOPS (Nacalai Tesque), pH 7, and then sonicated 5 times for 5 min each time in an ice−water bath. The mixture was centrifuged for 15 min at 8500 rpm. The precipitates were suspended in 50 mM MOPS. After addition of DNase I (5 μg /ml) and RNase A (20 μg /ml), the solution was incubated at 37 °C for 30 min to decompose DNA and RNA. Then sodium chloride was added to the solution to a final concentration of 1 M, and the mixture was again centrifuged for 10 min at 8500 rpm. The precipitates were suspended in 10 mL of 8 M urea solution [0.1 M Tris-HCl buffer (pH 8.6) containing 1 mM EDTA and 8 M urea], degassed by aspiration, purged with nitrogen, and reduced with 50 μL of mercaptoethanol at 40 °C for 1.5 h. TAPS (225 mg) was then added, and the reduction solution was incubated at 40 °C for 90 min to increase the solubility of the denatured lysozyme variants. The reaction mixture was dialyzed against distilled water. The dialysate was applied to a CM-Toyopearl 650M column (70 cm × 1.6 cm) equilibrated with 20 mM acetate buffer (pH 5) containing 4 M urea. The column was eluted with a gradient of 500 mL of 20 mM acetate buffer (pH 5) containing 4 M urea and 500 mL of the same buffer containing both 4 M urea and 1 M NaCl. Then the collected protein fraction was directly applied to a Cosmosil C18 column (1.2 cm × 1.6 cm) equipped for HPLC, which was equilibrated with 10% acetonitrile containing 0.1% TFA. The column was eluted with 40% acetonitrile containing 0.1% TFA, and the protein was collected. Lyophilized protein (0.5 mg) was dissolved in 2 mL of 6 M guanidine solution [0.1 M Tris-acetate buffer (pH 8.0) containing 6 M guanidine hydrochloride]. Mercaptoethanol (5 μL) was then added, and the solution was incubated at 40 °C for 1.5 h. The reduction mixture was applied to a μ-BONDASPHERE C4 column (150 mm × 3.9 mm) equilibrated with 20% acetonitrile containing 0.05% TFA. The column was eluted with a gradient of 50 mL of 30% acetonitrile containing 0.05% TFA and 50 mL of 40% acetonitrile containing 0.05% TFA at a flow rate of 0.1 mL/min. Each protein purified as described above was determined to be homogeneous by matrix-assisted laser desorption ionization (MALDI) time of flight (TOF)−mass spectrometry (MS) and SDS−PAGE analyses.

Evaluation of the Formation of Disulfide Bonds in Lysozyme Variants. The equilibrium disulfide formation reaction was carried out in 100 mM Tris-acetate, 0.1 mM EDTA, pH 8.0, at 22 °C in the presence of 0.1 mM GSSG[1] and 1.0 mM GSH with a protein concentration of 0.5 μM. The reaction was stopped at 240 min by adding acetic acid to 1 M, and the product was subjected to reversed phase HPLC on μ-BONDASPHERE C4 columns (150 mm × 3.9 mm, Waters Associates) at 40 °C in 0.05% TFA with a gradient of acetonitrile from 30% to 40%. The extent of the formation of disulfide bonds was expressed as the ratio of [Oxi] to [Red], which is obtained from the peak area on the chromatographic pattern of each lysozyme variant.

Calculation of the Decrease in Entropy in a Lysozyme Variant Containing a Pair of Cysteines. The decrease in entropy in each lysozyme variant by formation of a disulfide bond is calculated as $0.75\upsilon R[\ln(N/\upsilon + 3)]$, where N and υ correspond to the number of amino acid residues surrounded by a disulfide bond and twice the number of disulfide bonds, respectively (*18*).

RESULTS AND DISCUSSION

Preparation of 28 Lysozyme Variants Possessing a Pair of Cysteines. Hen lysozyme contains four disulfide bonds:

[1] Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; MD, mixed disulfide; Oxi, the oxidized variant in which one disulfide bond is formed in the lysozyme variant; [Oxi], the concentration of the oxidized form; Red, the reduced variant in which the pair of cysteines remained reduced; [Red], the concentration of the reduced form.

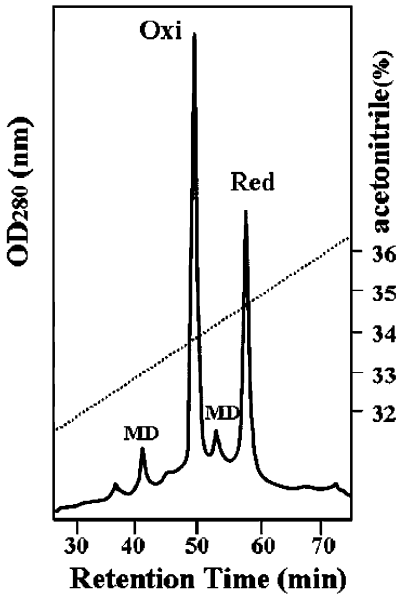

FIGURE 1: A typical pattern of the Oxi and Red forms of the Cys64−Cys80 lysozyme variant in the presence of 6 M guanidine hydrochloride on the reversed phase HPLC. The label MD indicates the glutathione-linked lysozyme variants.

Cys6−Cys127, Cys30−Cys115, Cys64−Cys80, and Cys76−Cys94. In the early stage of the folding of reduced lysozyme, it is possible that not only native disulfide bonds but also nonnative disulfide bonds form. Therefore, to examine the propensities for the formation of individual disulfide bonds in hen lysozyme, we prepared 28 lysozyme variants containing a pair of cysteines, in which the other cysteine residues are replaced by alanine residues.

Evaluation of the Extent of Disulfide Bond Formations in Lysozyme Variants Containing a Pair of Cysteines in the Presence of 6 M Guanidine Hydrochloride. A typical chromatogram for the Cys64−Cys80 lysozyme variant after oxidation of reduced lysozyme in the presence of 6 M guanidine hydrochloride is shown in Figure 1. The oxidized variant (denoted Oxi) and the reduced variant (denoted Red) were separated from each other. The pattern was highly reproducible. Red or Oxi peaks from each lysozyme variant were well separated from each other, and mixed disulfide peaks, which were derived from a lysozyme variant linked to one or two glutathione molecule(s) through a disulfide bond, were also separated from the Red or Oxi peaks in all other lysozyme variants (data not shown).

Recently, it has been reported that there is some structure in the denatured state of a protein (*19*). Specifically, we have demonstrated the presence of some hydrophobic clusters in the reduced and denatured state of hen lysozyme in the presence of 8 M urea at pH 2, which creates an extremely denatured condition (*20*). Therefore, to determine whether there is some structure in lysozyme in the presence of 6 M guanidine hydrochloride, which creates highly denatured condition, we used a series of lysozyme variants as a probe and measured the extent of disulfide bond formation in lysozyme variants in the presence of 6 M guanidine hydrochloride. With the exception of the Cys76/Cys80 lysozyme variant, the extent of disulfide bond formation in a lysozyme variant containing a pair of cysteines was proportional to the distance between cysteine residues. We can calculate the decrease in entropy of the polypeptide chain by introduction of disulfide bonds in lysozyme variants according to the equation of Flory (*18*), as described above in the Material and Methods section. Then we plotted the free energy change for the formation of disulfide bonds in a lysozyme variant containing a pair of cysteines, with the exception of the Cys76/Cys80 lysozyme variant, in the presence of 6 M guanidine hydrochloride against the theoretical free energy change derived from the decrease in entropy of the polypeptide chain (open diamonds in Figure 2). There was a good correlation between them. This showed that the denatured state of hen lysozyme in the presence of 6 M guanidine hydrochloride adopts a randomly coiled structure. This might be a most beautiful expression of chain entropy, and at the same time, this showed the reliability of this analysis. On the other hand, as for the Cys76/Cys80 lysozyme variant, the extent of disulfide bond formation ran off the correlation between the extent of disulfide bond formation and distance of cysteine residues (Figure 2). It is known that the formation of the disulfide bond is restricted due to the steric hindrance when the distance between cysteine residues is too short (*22*). This may be the case with the Cys76/Cys80 lysozyme variant. Thus, we exclude this SS bond from present discussions. Anyway, we were able to demonstrate that no residual structure was present in reduced hen lysozyme in the presence of 6 M guanidine hydrochloride.

α-Lactalbumin is known to have primary and tertiary structures similar to hen lysozyme. In their study using α-lactalbumin variants containing two native disulfide bonds, Wu et al. suggested that the formations of disulfide bonds in α-lactalbumin in the presence of 6 M guanidine hydrochloride were controlled by the random polypeptide chain entropy (*21*). This agrees with the present results. However, the information on the α-lactalbumin is limited by the use of two native disulfide bond formations. In the present study, we could obtain detailed information on the denatured state of hen lysozyme using 28 lysozyme variants containing a pair of cysteines. In this way, we were able to conclusively demonstrate that reduced hen lysozyme adopted a randomly coiled structure in the presence of 6 M guanidine hydrochloride.

Evaluation of the Extent of Disulfide Bond Formations in Lysozyme Variants Containing a Pair of Cysteines in Aqueous Solution. Figure 2 shows a plot of the free energy changes (ΔG's shown in Figure 2) in the formations of the disulfide bonds in lysozyme variants in aqueous solution against the theoretical free energy changes derived from the decreases in entropies of the polypeptide chain of lysozyme (open circles). Even in aqueous solution, the formations of disulfide bonds are governed by the chain entropies to some extent. However, all the disulfide bonds in aqueous solution formed more efficiently than those in the presence of 6 M guanidine hydrochloride. The exchange rate constant between a cysteine residue and a GSSG in aqueous solution was reported to be almost identical to that in the solution containing concentrated denaturant (*23*). Therefore, we concluded that the denatured lysozyme in aqueous solution had a compact cluster due to the interactions between hydrophobic residues within the core of the protein in accord with the evidence of the previous NMR experiment (*20*), and we presumed that compact clusters of this type allow each disulfide bond to be formed easily.

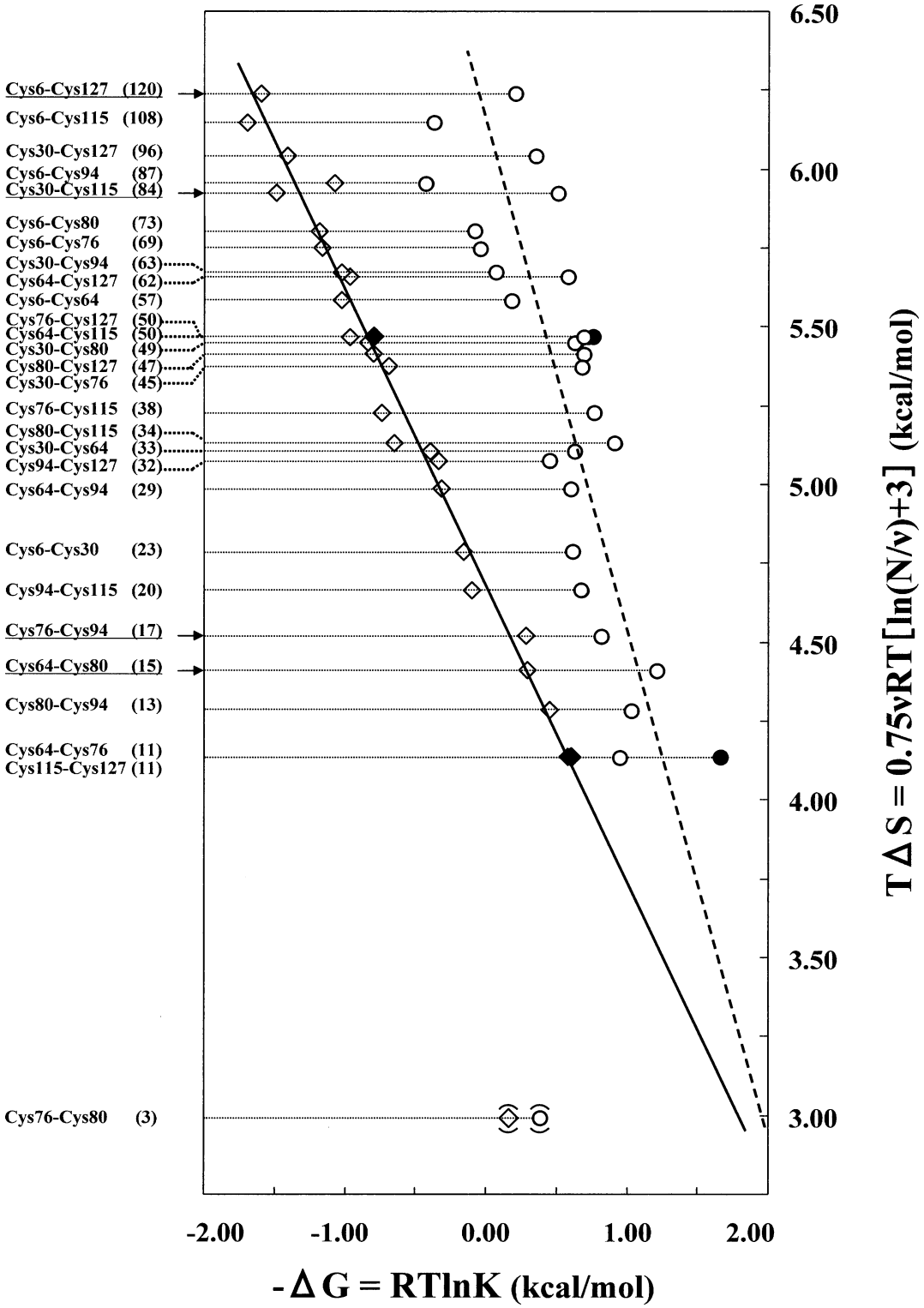

FIGURE 2: The free energy changes for the formations of disulfide bonds in lysozyme variants in the presence of 6 M guanidine hydrochloride (◇) and those in aqueous solution (○) against the theoretical free energy changes derived from the decrease in entropy of the polypeptide chain of lysozyme at pH 8 and 22 °C. The lysozyme variants underlined indicate pairs of cysteines for native disulfide bonds. Points for Cys76/Cys127 and Cys64/Cys76 are denoted by filled marks to clarify the figure. Data points with parentheses are not included due to steric hindrance when we draw lines.

As for the four native disulfide bonds (Cys6−Cys127, Cys30−Cys115, Cys64−Cys80, and Cys76−Cys94), they did not always form preferentially. To compare ΔG for the formation of the disulfide bond against all the variants, we calculated the difference in the ΔG value for the formation of the disulfide bond from the best fit line (dotted line) at the particular points (open circles). The ΔG for the formation of the disulfide bond in the Cys64/Cys76 lysozyme variant was the highest in all variants. The difference from the best fit line was about 0.4 kcal/mol, indicating that the Cys64−Cys76 bond formed most preferentially in the early stage of the folding of reduced lysozyme than other disulfide bonds. The Cys6/Cys94 lysozyme variant, in contrast, was the lowest in the free energy difference and the difference was

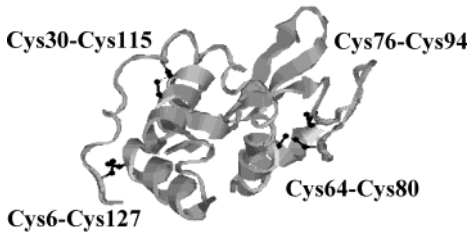

FIGURE 3: The three-dimensional structure of hen egg white lysozyme. Four native disulfide bonds are shown by stick model. The structure of lysozyme mainly consists of two domains, α-domain and β-domain. The eight cysteines are geographically distributed into two sets, according to whether they lie in one lobe or the other located on either side of the active site cleft. Cysteines 64, 76, 80, and 94 lie in the β-domain, and the other four cysteines lie in a separate α-domain.

about 0.6 kcal/mol, indicating that the Cys6−Cys94 bond formed much more rarely in the early stage of the folding of reduced lysozyme than the other disulfide bonds.

Here, we furthermore note that in native lysozyme, the eight cysteine residues geographically fall into two sets, according to whether they lie in one lobe or the other located on either side of the active site cleft shown as Figure 3. Cysteine residues 64, 76, 80, and 94 lie in one lobe, and the other four cysteine residues lie in the other lobe. We consider 10 data points at the top of Figure 2 where cysteine residues are further than 50 residues apart in the primary sequence. These cysteine pairs were least likely to come close by random motions. Among these pairs of cysteines, 3 out of 4 cases that generated higher values than the best fit line lay in the same lobe, and 5 out of 6 cases that generated lower values than the best fit line lay in two different lobes. These results imply that nativelike folding in the reduced protein might enhance formation of intralobe disulfide bonds between two cysteine residues in the same lobe, while interlobe disulfide bond formations between two cysteine residues in different lobes might be suppressed. Two pairs of cysteines, Cys64−Cys76 and Cys6−Cys94, which were most apart from the best fit line might be representative examples for these cases. Thus, the scattering of the ΔG's for the formations of the disulfide bonds among all lysozyme variants suggested that the nonrandom folding occurred in the early stage of the folding of reduced lysozyme.

Van den Berg et al. (*15*) suggested that no prominent disulfide bonds formed before the formation of the folding intermediate with three disulfide bonds. On the other hand, in the present paper, we showed that nonrandom folding occurred in the early stage of the folding of reduced lysozyme. Especially formation of Cys64−Cys76 should easily occur in the early stage of folding of the reduced lysozyme because the ΔG for the formation of the disulfide bond of the Cys64/Cys76 lysozyme variant was the largest in the free energy changes among all the variants. Thus, the preferential formation of Cys64−Cys76 might facilitate the formations of Cys64−Cys80 and Cys76−Cys94 by exchanges, which might form earlier than the other disulfide bonds (*24*,*25*), indicating that there is a folding pathway involving the formations of the cysteines in the early stage of the folding of reduced lysozyme.

CONCLUDING REMARKS

We prepared 28 species of lysozyme variants containing a pair of cysteines and examined the interaction of the residues in the presence or absence of concentrated denaturant. We showed that there was no prominent interaction between amino acid residues in the presence of 6 M guanidine hydrochloride. Even in aqueous solution, the formations of disulfide bonds were governed by the chain entropies to some extent. However, all the disulfide bonds in aqueous solution formed more efficiently than those in the presence of 6 M guanidine hydrochloride. This means that the reduced lysozyme has a somewhat compact structure and that nonrandom folding occurred in the early stage of the folding of reduced lysozyme.

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REFERENCES

1. Lopez De La Paz, M., Goldie, K., Zurdo, J., Lacroix, E., Dobson, C. M., Hoenger, A., and Serrano, L. (2002) De novo designed peptide-based amyloid fibrils, *Proc. Natl. Acad. Sci. U.S.A. 99*, 16052−16057.
2. Gillespie, B., Vu, D. M., Shah, P. S., Marshall, S. A., Dyer, R. B., Mayo, S. L., and Plaxco, K. W. (2003) NMR and temperature-jump measurements of de novo designed proteins demonstrate rapid folding in the absence of explicit selection for kinetics, *J. Mol. Biol. 330*, 813−819.
3. Thomas, B. J., Qu, B.-H., and Pederson, P. L. (1995) Defective protein folding as a basis of human disease, *Trend Biochem. Sci. 20*, 456−459.
4. Kim, P. S., and Baldwin, R. L. (1982) Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding, *Annu. Rev. Biochem. 51*, 459−489.
5. Goldenberg, D. P., and Creighton, T. E. (1984) Folding pathway of a circular form of bovine pancreatic trypsin inhibitor, *J. Mol. Biol. 179*, 527−545.
6. Weissman, J. S., and Kim, P. S. (1991) Reexamination of the folding of BPTI: predominance of native intermediates, *Science 253*, 1386−1393.
7. Ruoppolo, M., and Freedman, R. B. (1994) Protein-S−S-glutathione mixed disulfides as models of unfolded proteins, *Biochemistry 33*, 7654−7662.
8. Ruoppolo, M., Freedman, R. B., Pucci, P., and Marino, G. (1996) Glutathione-dependent pathways of refolding of RNase T1 by oxidation and disulfide isomerization: catalysis by protein disulfide isomerase, *Biochemistry 35*, 13636−13646.
9. Shin, H. C., Narayan, M., Song, M. C., and Scheraga, H. A. (2003) Role of the [65−72] disulfide bond in oxidative folding of bovine pancreatic ribonuclease A, *Biochemistry 42*, 11514−11519.
10. Rothwarf, D. M., Li, Y. J., and Scheraga, H. A. (1998) Regeneration of bovine pancreatic ribonuclease A: identification of two nativelike three-disulfide intermediates involved in separate pathways, *Biochemistry 37*, 3760−3766.
11. Iwaoka, M., Juminaga, D., and Scheraga, H. A. (1998) Regeneration of three-disulfide mutants of bovine pancreatic ribonuclease A missing the 65−72 disulfide bond: characterization of a minor folding pathway of ribonuclease A and kinetic roles of Cys65 and Cys72, *Biochemistry 37*, 4490−4501.
12. Peng, Z. Y., Wu, L. C., and Kim, P. S. (1995) Local structural preferences in the α-lactalbumin molten globule, *Biochemistry 34*, 3248−3252.
13. Wu, L. C., and Kim, P. S. (1998) A specific hydrophobic core in the alpha-lactalbumin molten globule, *J. Mol. Biol. 280*, 175−182.
14. Anderson, W. L., and Wetlaufer, D. B. (1976) The folding pathway of reduced lysozyme, *J. Biol. Chem. 251*, 3147−3153.
15. van den Berg, B., Chung, E. W., Robinson, C. V., Mateo, P. L., and Dobson, C. M. (1999) The oxidative refolding of hen

lysozyme and its catalysis by protein disulfide isomerase, *EMBO J. 18*, 4794−4803.
16. Tachibana, H. (2000) Propensities for the formation of individual disulfide bonds in hen lysozyme and in the size and stability of disulfide-associated submolecular structures, *FEBS Lett. 480*, 175−178.
17. Mine, S., Ueda, T., Hashimoto, Y., and Imoto, T. (1997) Improvement of the refolding yield and solubility of hen egg-white lysozyme by altering the Met residue attached to its N-terminus to Ser, *Protein Eng. 11*, 1333−1338.
18. Flory, P. J. (1956) Theory of elastic mechanisms in fibrous proteins, *J. Am. Chem. Soc. 78*, 5222−5235.
19. Lietzow, M. A., Jamin, M., Jane Dyson, H. J., and Wright, P. E. (2002) Mapping long-range contacts in a highly unfolded protein, *J. Mol. Biol. 322*, 655−662.
20. Klein-Seetharaman, J., Oikawa, M., Grimshaw, S. B., Wirmer, J., Duchardt, E., Ueda, T., Imoto, T., Smith, L. J., Dobson, C. M., and Schwalbe, H. (2002) Long-range interactions within a nonnative protein, *Science 295*, 1719−1722.
21. Wu, L. C., Peng, Z. Y., and Kim, P. S. (1995) Bipartite structure of the alpha-lactalbumin molten globule, *Nat. Struct. Biol. 2*, 281−286.
22. Zhang, R. M., and Snyder, G. H. (1991) Factors governing selective formation of specific disulfides in synthetic variants of α-conotoxin, *Biochemistry 30*, 11343−11348.
23. Goto, Y., and Hamaguchi, K. (1981) Formation of the intrachain disulfide bond in the constant fragment of the immunoglobulin light chain, *J. Mol. Biol. 146*, 321−340.
24. Ueda, T., Ohkuri, T., and Imoto, T. (1996) Identification of the peptide region that folds native conformation in the early stage of the renaturation of reduced lysozyme, *Biochem. Biophys. Res. Commun. 228*, 203−208.
25. Ohkuri, T., Ueda, T., Tsurumaru, M., and Imoto, T. (2001) Evidence for an initiation site for hen lysozyme folding from reduced form using its dissected peptide fragments, *Protein Eng. 14*, 829−833.

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