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Identification of the Peptide Region That Folds Native Conformation in the Early Stage of the Renaturation of Reduced Lysozyme

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We prepared three peptide fragments (fg.59-105, fg.63-105 and fg.64-105) by the BrCN cleavage of mutant lysozymes where Ile58, Trp62 and Trp63 were mutated to Met, respectively. From the analysis of formation of the disulfide bonds among Cys64, Cys76, Cys80 and Cys94 in the renaturation of each peptide fragment from the reduced form, Trp62 and Trp63 were required for the effective formation of two disulfide bonds. Especially, Trp62 was found to be involved in the correct formation of the disulfide bonds. © 1996 Academic Press, Inc.

Kim and Baldwin (1) have demonstrated that the analyses of the early events of the protein folding are effective for the elucidation of the mechanism of protein folding. The kinetical analysis for the early stage of the protein folding by means of circular dichroism or NMR spectroscopy with pulse-labeling technique would give us many information on the mechanism of protein folding (2-4). On the other hand, to dissect protein is a method to know how some parts in the whole protein contribute in the protein architecture. For example, if some peptide region folds without the help of the other peptide region, the peptide region in the whole protein should fold in the early stage of the protein folding.

Hen egg white lysozyme has four disulfide bonds, Cys6-Cys127, Cys30-Cys115, Cys64-Cys80 and Cys76-Cys94 (5). When reduced lysozyme was renaturated by sulfhydryl-disulfide interchange reactions, two disulfide bonds, Cys64-Cys80 and Cys76-Cys94, which are located inside of the molecule formed earlier than the other ones (6). With respect to protein folding, it should be reasonable to focus our attention on the peptide region around these two cystines. It was reported that the peptide fragment between Lys13 and Homoserine 105 (fg.13-105) could renaturate to the peptide structure that has the affinity to lysozyme substrate-immobilized column (7). However, there was no experimental data for the renaturation of the shorter peptide than the fg.13-105 from the reduced form. Therefore, in this report, we prepared three shorter peptide fragments than the fg.13-105 and identified the peptide region that folded in the early stage of the renaturation of reduced lysozyme.

MATERIALS AND METHODS

Expression and purification of mutant lysozymes. We prepared mutant lysozymes where Ile58, Trp62 and Trp63 in hen lysozyme were mutated to Met, respectively, according to the methods of Hashimoto *et al.* (8). These mutations in the lysozyme gene were confirmed by DNA sequencing. These yeast *Saccharomyces cerevisiae* AH22 transformants

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Abbreviations: fg.13-105, a peptide fragment between Lys 13 and Homoserine 105; fg.59-105, a peptide fragment between Asn59 and Homoserine 105; fg.63-105, a peptide fragment between Trp63 and Homoserine 105; fg.64-105, a peptide fragment between Cys64 and Homoserine 105; NMR; nuclear magnetic resonance, RP-HPLC, reversed phase-high performance liquid chromatography.

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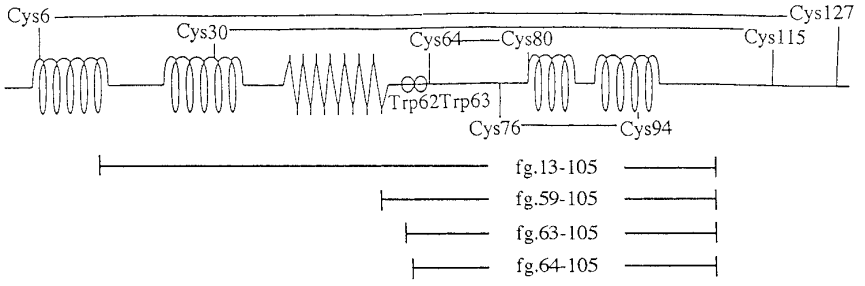

FIG. 1. Secondary structures and the positions of cystines in hen lysozyme. ( ) and ( ) indicate α-helix and β-sheet, respectively.

prepared were cultivated at 30°C for 125 h to express and secrete the mutant lysozyme as described in the literature (8). The purification of mutant lysozymes were carried out according to our previous report (9).

Preparation of peptide fragments. Five milligrams of mutant lysozymes were dissolved in 1 ml of 6M guanidine hydrochloride-1N HCl, respectively. To each solution, 100 mg of BrCN was added and stirred for 20h at room temperature under the dark. After the evaporation of BrCN, each solution was redissolved in 1 ml of 10% aqueous acetic acid and applied to the column of Sephadex G-75 (1.5 × 90 cm). Each desired peptide (Asn59-Homoserine 105, Trp63-Homoserine 105 and Cys64-Homoserine 105) could be completely separated from the other peptide fragments under the condition. The preparation of these fragments were confirmed from the amino acid composition after acid hydrolysis of the peptide fragment with an Hitach L-8500 amino acid analyzer.

Renaturation of the reduced peptide fragments and analysis of the formation of disulfide bonds. Reduction and renaturation of reduced peptide fragments was carried out in 0.2 M Tris-acetate buffer (pH 8.0) at 37°C using 0.3 μM peptide fragments according to the method of Ueda *et al.* (10). After the reaction of each renaturated peptide fragments with *N*-ethylmaleimide (10 mM) at pH 5.5, the pH of each reaction mixture was lowered to 3 and dialyzed against 10% aqueous acetic acid using dialyzing tube (cutoff 3,500). Then, each dialysate was purified using RP-HPLC according to the literature (10). Analysis of the disulfide bond of each renaturated peptide fragment was carried out using two step protease digestions according to the literature (10).

Reduction of the tryptic peptides. To the digestion mixture (1 ml) of the peptide fragment with trypsin, 5 ml of 2-mercaptoethanol was added and incubated at 40°C for 2h.

RESULTS AND DISCUSSION

In the fg.13-105 (Figure 1), there were two helical structures (from residue 25 to 35 and from residue 88 to 98), one β-sheet (from residues 45 to 55), two cystines (64-80, 76-94) and one half cystine (Cys 30). The synthetic peptide (residue 41 and 60) including the β-sheet was apt to oligomerize (11). On the other hand, the modification of Trp62 affected the renaturation of reduced lysozyme (10,12). Therefore, we prepared three peptide fragments, fg.59-105, fg.63-105 and fg.64-105, and we analyzed the formation of disulfide bond among four cysteines in each fragment renaturated from reduced form. The analysis of disulfide bonds among Cys64, Cys76, Cys80 and Cys94 was carried out in two steps according to the method of our previous paper (10). Figure 2A shows RP-HPLC pattern of the peptide derived from tryptic digestion of the renaturated peptide fragment 59-105. The elution position of peak A was identical to that of the peptides Trp62-Arg68 plus Asn74-Lys96 as was shown in the previous paper (10). The amino acid composition of peak A indicated that the peak A was the peptide Trp62-Arg68 plus Asn74-Lys96 where these peptides covalently connected through disulfide bonds (data not shown). Moreover, in order to neglect the possibility that peak A is not a complex of these peptides, RP-HPLC pattern of the reducted products of the tryptic peptides was analyzed (Figure 2B). After the reduction, peak A disappeared and two new peaks appeared. The former peak was identified to be the peptide Trp62-Arg68 and the latter one was identified to be the peptide Asn74-Lys96 from the respective amino acid composition (data not shown). The result indicates that the original peak A was not a complex of the peptides Trp62-Arg68 and Asn74-Lys96. This was supported by the result that peak A did

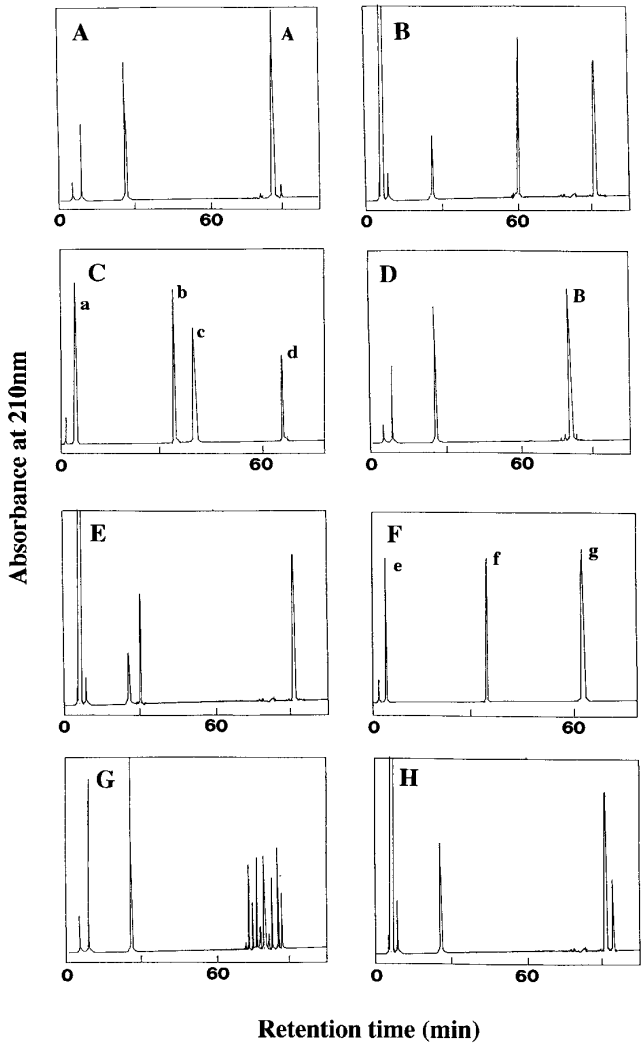

FIG. 2. RP-HPLC of digests of the renaturated fragment with protease on a column (Wakosil 5C18, 4.6 × 250 mm). The column was eluted with a gradient of 40 ml of 1% acetonitrile and 40 ml of 40% acetonitrile both containing 0.1% HCl at a flow rate of 0.6 ml/min. A, derived from the renaturated fg.59-105 digested with trypsin; B, the peptides in Figure 2A after reduction by 2-mercaptoethanol; C, derived from peak A in Figure 2A digested with both α-chymotrypsin and prolylendopeptidase; D, derived from the renaturated fg.63-105 digested with trypsin; E, the peptides in Figure 2D after reduction by 2-mercaptoethanol; F, derived from peak B in Figure 2D digested with both α-chymotrypsin and prolylendopeptidase; G, derived from the renaturated fg.64-105 digested with trypsin; H; the peptides in Figure 2G after reduction by 2-mercaptoethanol.

not include any free cysteine since there was no carboxymethylated cysteine in the sample where peak A was incubated with monoiodoacetic acid in 8M urea solution (data not shown).

The amino acid sequence of the peptide between Asn59 and Homoserine 105 in native lysozyme is shown in Figure 3. For further investigation of disulfide bonds in the peak A, the peptide was isolated, lyophilized, and digested with α-chymotrypsin and prolylendopeptidase. RP-HPLC pattern of the digested peptide is shown in Figure 2C. In order to examine the formation of disulfide bonds, each peptide on RP-HPLC was isolated. The presence of Trp was examined by monitoring the elution of each peptide at 280 nm and the amino acid

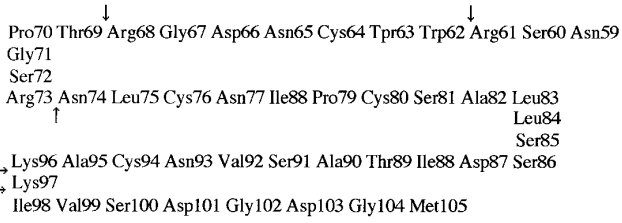

FIG. 3. Amino acid sequence of fg.59-105. The arrow indicates the cleaved site with trypsin.

composition after the peroxidation of each peptide was analyzed. As was shown in Table 1, the correct formation of two cystines was confirmed. Therefore, β-sheet region and one half cystine (Cys30) was not involved in the correct formation of disulfide bonds (Cys64-Cys80 and Cys76-Cys94).

Similarly, Figure 2D shows RP-HPLC pattern of the peptide derived from the tryptic digestion of the renaturated fg. 63-105. The amino acid composition indicated that peak B was the peptide Trp63-Arg68 plus Asn74-Lys96 where these peptides covalently connected through disulfide bonds (data not shown). This is consistent with the observation that peak B eluted earlier than the peptide A due to the truncation of Trp62 from the peptide Trp62-Arg68. Moreover, in order to neglect the possibility that peak B is not a complex of these peptides, RP-HPLC pattern of the reduced products of the tryptic peptides was analyzed (Figure 2E). After the reduction, peak B disappeared and two new peaks appeared. The former peak was identified to be the peptide Trp63-Arg68 and the latter one was identified to be the peptide

TABLE 1
Amino Acid Composition of Peptides after Acid Hydrolysis in Fig. 2C

	Peak a		Peak b		Peak c		Peak d	
Amino acide	Calc.	Theory	Calc.	Theory	Calc.	Theory	Calc.	Theory
Asp	2.0	2	0.9	1	2.8	3	0	0
Thr	0	0	1.0	1	0	0	0	0
Ser	0.8	1	2.8	3	0	0	0	0
Glu	0	0	0	0	0	0	0	0
Gly	1.1	1	0	0	0	0	0	0
Ala	1.0	1	1.0	1	1.0	1	0	0
Val	0	0	1.0	1	0	0	0	0
Met	0	0	0	0	0	0	0	0
Ile	0	0	0.9	1	0.8	1	0	0
Leu	0	0	1.0	1	0.9	1	0	0
Tyr	0	0	0	0	0	0	0	0
Phe	0	0	0	0	0	0	0	0
Lys	0	0	0	0	1.0	1	0	0
His	0	0	0	0	0	0	0	0
Arg	0.8	1	0	0	0	0	0	0
Pro	0	0	0	0	1.0	1	0	0
Cys-SO_3H	2.0	2	0	0	2.0	2	0	0
Trp	−	−	−	−	−	−	++	++
Assigned residue NO.	64–68 | 80–82		84–92		74–79 | 96–93		62–63	

TABLE 2
Amino Acid Composition of Peptides in Fig. 2F after Acid Hydrolysis

	Peak e		Peak f		Peak g	
Amino acid	Calc.	Theory	Calc.	Theory	Calc.	Theory
Asp	1.0	1	0.9	1	3.8	4
Thr	0	0	1.0	1	0	0
Ser	0.8	1	2.8	3	0	0
Glu	0	0	0	0	0	0
Gly	0	0	0	0	1.0	1
Ala	2.0	1	1.0	1	0	0
Val	0	0	1.0	1	0	0
Met	0	0	0	0	0	0
Ile	0	0	0.9	1	0.8	1
Leu	0	0	0	0	0.9	1
Tyr	0	0	0	0	0	0
Phe	0	0	0	0	0	0
Lys	1.0	1	0	0	0	0
His	0	0	0	0	0	0
Arg	0	0	0	0	0.9	0
Pro	0	0	0	0	1.0	1
Cys-SO_3H	2.0	2	0	0	2.0	2
Trp	−	−	−	−	+	+
Assigned residue NO.	80–82 | 96–93		85–92		63–68 | 74–79	

Asn74-Lys96 from the respective amino acid composition (data not shown). The result indicates that the peak B was not a complex of the peptides Trp63-Arg68 and Asn74-Lys96. This was supported by the result that original peak B did not include any free cysteine since there was no carboxymethylated cysteine in the sample where peak B was incubated with monoiodoacetic acid in 8M urea solution (data not shown). Then, the analysis of the disulfide bond of the peak B was carried out by protease digestion described above (Figure 2F). The presence of Trp was examined by monitoring the elution of each peptide at 280nm. From amino acid composition after the peroxidation of each peptide (Table 2), the predominant formation of non-native disulfide bonds (Cys64-Cys76 and Cys80-Cys94) was found to occur. Therefore, the peptide Asn 59-Trp62 was concluded to be involvement in the correct formation of disulfide bonds. In the previous data, we suggested that the interaction between the hydrophobic side of the helix and Trp 62 and/or Trp63 were important in the early stage of the folding of reduced lysozyme (9). In the folded state, Trp63 is closely located near the α-helix 88-98 but Trp62 is not (13). However, since there is generally the interactions between the amino acid side chains in the sequence of Trp-Trp or Trp-Tyr (14), the interaction between Trp62 and Trp63 must be present in the reduced form. Namely, the orientation of Trp63 can be restricted by the presence of Trp62 even in the reduced form. The interaction between them would lead these cystines (Cys64, Cys76, Cys80 and Cys94) to the formation of correct disulfide bonds (Cys64-Cys80 and Cys76-Cys94) in the fg. 59-105. On the other hand, in the fg.63-105, as the residues Asn59-Trp62 (especially Trp62) were truncated from the fragment, Trp63, which is not restricted, may freely interact with the hydrophobic side of helix 88-98. As the result, the non-native disulfide bonds (Cys64-Cys76 and Cys80-Cys94) formed predominantly.

Figure 2G shows RP-HPLC pattern derived from tryptic digestion of the renaturated fg. 64-

105. Several peaks were observed in the latter part on RP-HPLC. For comparison, the RP-HPLC pattern of the peptide after reduction of the tryptic peptides is shown in Figure 2H. There appeared a major peak and a minor peak in the latter part on RP-HPLC. From the amino acid composition (data not shown), these peptides were derived from the peptide Asn74-Lys96 while the minor peak was one reacted with *N*-ethylmaleimide which was employed for the trapping of the renaturated peptides. Therefore, the complicated pattern in the latter part on RP-HPLC in Figure 2E was majorly due to the formation of the scrambled disulfide bonds, which somewhat contains incomplete disulfide bonds. Namely, further truncation of Trp63 from the fg. 63-105 may lead four cysteines to the formation of disordered disulfide bonds. The result was consistent with the previous our observation that Trp62 and/or Trp63 interacted with the helix 88-98 in the early stage of the folding of reduced lysozyme (9).

We summarize our results as follows 1) we identified the peptide region to lead four cystines, which forms in the early stage of the renaturation of reduced form, to form native disulfide bonds, 2) Trp62 may be involved in the correct formation of these disulfide bonds from reduced form, 3) The interaction between Trp62 and/or Trp63 and the helix 88-98 (9) was confirmed to be essential in the effective formation of these disulfide bonds from reduced form. The result obtained here would be significant for understanding the early event in the renaturation of reduced lysozyme but also for *de novo* design of functional proteins since it gave information of structural unit. Moreover, some models for the mechanism of protein folding have been already submitted, such as jigsaw puzzle model, nucleation-rapid growth model and so on (15). In lysozyme, there is few discussion which model may be appropriate based on experimental evidence. The identification of the peptide region folds in the early stage of the renaturation of reduced lysozyme must be a key point to elucidate the mechanism in the folding of lysozyme from reduced form experimentally.

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