Study on the Early Events in Folding of Hen Lysozyme from Reduced Form by Using of Mutants or Peptide Fragments

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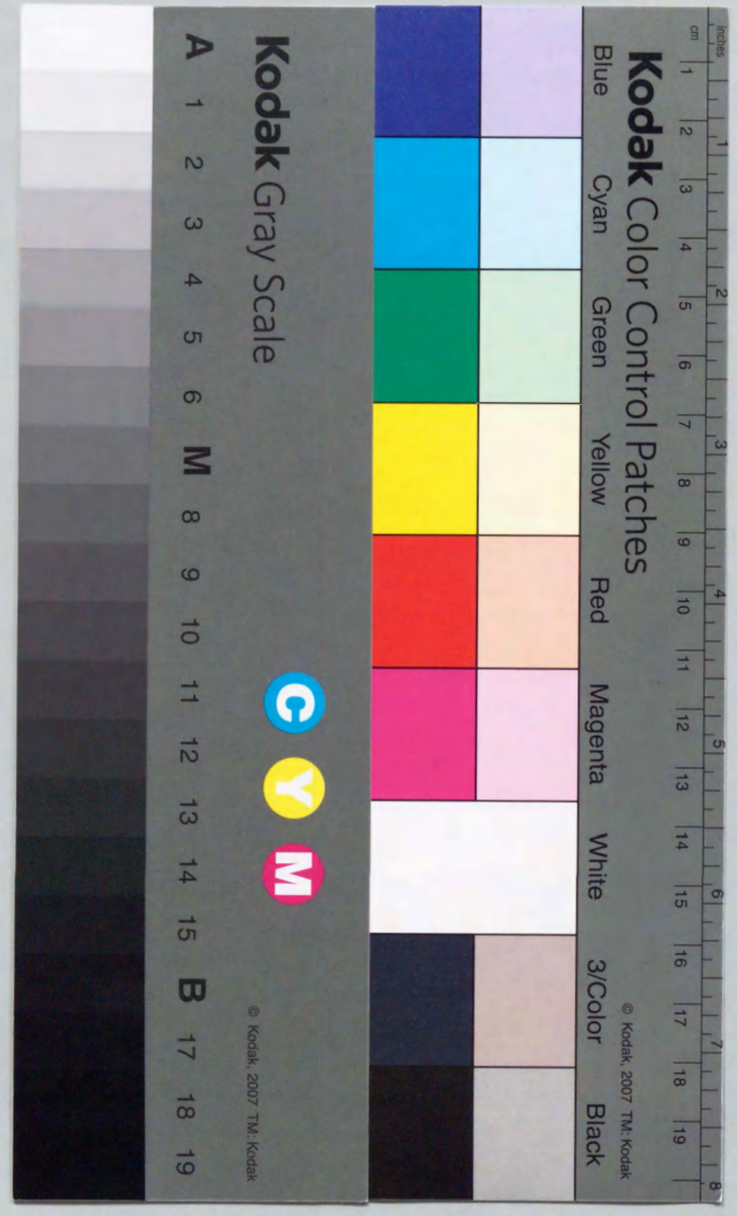

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TAKATOSHI OHKURI

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Abbreviations

AEMTS, 2-amioethyl- methanthiosulfonate
ANS, 1-anilinonaphthalene-8-sulfonic acid
BPTI, bovine pancreatic trypsin inhibitor
BrCN, cyanogen bromide
CD, circular dichroism
CM-lysozyme, S-carboxymethylated lysozyme
Gdn-HCl, guanidine hydrochloride
GSSG, oxidized glutathione
IPTG, isopropyl β-D-thiogalactoside
NMR, nuclear magnetic resonance
RP-HPLC, reverse phase high-performance liquid chromatography
TPCK-trypsin, L-(tosylamino 2-phenyl) ethyl chloromethyl-trypsin
UV, ultraviolet

The lysozyme derivative

I 58 M lysozyme, a mutant lysozyme where Ile 58 is mutated to Met; **W 62 M lysozyme,** a mutant lysozyme where Trp 62 is mutated to Met; **W 63 M lysozyme,** a mutant lysozyme where Trp 63 is mutated to Met; **C 6 A C 30 A C 115 A C 127 A lysozyme,** a mutant lysozyme where Cys 6, Cys 30, Cys 115, and Cys 127 are simultaneously mutated to Ala; **W 62 G lysozyme,** a mutant lysozyme where Trp 62 is mutated to Gly; **W 62 N lysozyme,** a mutant lysozyme where Trp 62 is mutated to Asn; **W 62 H lysozyme,** a mutant lysozyme where Trp 62 is mutated to His; **W 62 F lysozyme,** a mutant lysozyme where Trp 62 is mutated to Phe; **CaB lysozyme,** a mutant lysozyme where the amino acid residues between position 82 and position 91 in hen lysozyme are mutated to Lys-Phe-Leu-Asp-Asp-Asp-Ile-Thr-Asp-Asp corresponding to the sequence of a Ca^{2+} binding loop region in human α-lactalbumin; **fg. 59 ~ 105,** peptide fragment between position 59 and 105; **fg. 63 ~ 105,** peptide fragment between position 63 and 105; **fg. 64 ~ 105,** peptide fragment between position 64 and 105; **fg. 1 ~ 58 + fg. 106 - 129,** peptide fragment between position 1 and 58 covalenty connected to peptide fragment between position 106 and 129 by disulfide bonds; **C 64 A C 76 A C 80 A C 94 A fg. 59 ~ 105,** peptide fragment between position 59 and 105 where Cys 64, Cys 76, Cys 80, and Cys 94 are simultaneously mutated to Ala; **W 62 G fg. 59 ~ 105,** peptide fragment between position 59 and 105 where Trp 62 is mutated to Gly.

CONTENTS

GENERAL INTRODUCTION

Anfinsen (1973) showed by the folding experiment of unfolded ribonuclease A that the information required to define the tertiary structure was encoded in the amino acid sequence. Since then, it has been confirmed that many proteins can spontaneously fold from their unfolded states, primary structure, *in vitro*. However, it is still not clear how proteins acquire their tertiary structures from their primary structures. Recently, a lot of primary sequences derived from human genome have been determined. If the principle of protein folding is clear, the functions of proteins whose primary sequences are determined by gene engineering technique will be easily analyzed and the possible designs of novel functional proteins will make a rapid progress. Therefore, in these meanings, the investigation to elucidate the protein folding mechanism will be significant. Moreover, Chothia (1992) found that folding patterns of proteins were limited to approximately 1000. The finding led us to the conclusion that we could obtain a general rule of protein folding by accumulating the information of the folding process of a large number of proteins, resulting in setting spurs to the investigation of protein folding in these days.

Hen lysozyme is an enzyme which has been analyzed its tertiary structure using X-ray crystallography for the first time of enzymes (Blake *et al*, 1967), and the structure-function relationships of which are well known. Hen lysozyme has four disulfide bonds Cys 6 - Cys 127, Cys 30 - Cys 115, Cys 64 - Cys 80, and Cys 76 - Cys 94. Its tertiary structure is divided into two domains, α-domain consisting of α-helix (4 ~ 15, 24 ~ 36, 88 ~ 98, and 108 ~ 115) and β-domain consisting of β-sheet (44 ~ 60) (Smith *et al*, 1993). The folding process of lysozyme under non-reduced condition have monitored by a wide variety of methods, including near- and far-UV CD (Caffotte et al, 1992; Radford et al, 1992), absorbance (Kato et al, 1981; Denton and Scheraga, 1994), inhibitor binding (Kato et al, 1981; Itzaki et al, 1994). And, Dobson's group at Oxford Univ.,

has reported that the formation of α-domain is earlier than β-sheet on the folding of lysozyme under non-reduced condition by analysis using pulse labeling NMR (Radford *et al*, 1992). As shown above, the information of folding process of lysozyme under non-reduced condition has been considerably accumulated, but its folding process from reduced form is little clear. The analysis of folding process from reduced form would be important because proteins fold from reduced form in living cells. Therefore, in this thesis, toward the elucidation of the general rule of protein folding, I have purposed for studying the folding process of hen lysozyme from reduced form, especially the early events in the folding where the information of protein folding could be obtained effectively (Kim and Baldwin, 1982).

To examine the folding from reduced protein, analyses of the formation of the disulfide bond have been widely used (Creighton, 1978; Weissman and Kim, 1991; Pace and Creighton, 1986; Schonbrunner and Schmid, 1992; Chatrenet and Chan, 1992). However, there was no method of qualitative and quantitative analysis of four disulfide bond formations in hen lysozyme. Therefore, in CHAPTER I, I have developed a novel method for the fragmentation of hen lysozyme into the peptides containing a single disulfide bond each.

Anderson and Wetlaufer (1976) analyzed the formation of disulfide bonds by trapping sulfhydryl-disulfide interchange reactions by alkyl reagents in the course of folding from reduced lysozyme, and reported that two cystines derived from Cys 64 - Cys 80, Cys 76 - Cys 94 formed earlier than the other cystines in the folding of the reduced lysozyme. However, they neither separated the intermediates nor identified the region formed in the early stage of folding. Therefore, in CHAPTER II, I have tried to analyze the disulfide bonds formed earlier than the other cystines by using the peptide fragments. As a result, I found that the minimum region for formation of correct disulfide bonds (Cys 64 - Cys 80, Cys 76 - Cys 94) was the region 59 ~ 105, and that Trp 62 was deeply involved in the correct formation of these disulfide bonds.

In CHAPTER II, I showed that the peptide fragment 59 ~ 105 of hen lysozyme formed the correct disulfide bond from reduced form without the help of the other peptide region. However, I do not refer to whether the formation of the peptide region 59 ~ 105 is involved in the folding process to acquire active structure of lysozyme Therefore, in CHAPTER III, I analyzed the involvement by association experiment between peptide fragments and by using the mutant lysozyme. As a result, it was suggested that the formation of the peptide fragment 59 ~ 105 is involved in the formation of active structure in the folding process of lysozyme and the formation of peptide region 59 ~ 105 in lysozyme was cue in the folding process of reduced lysozyme. Moreover, Trp 62 and two disulfide bond (Cys 64 - Cys 80 and Cys 76 - Cys 94) were essential to the maintenance of the structure of peptide region 59 ~ 105.

Alpha-lactalbumin is highly homologous to lysozyme and has a Ca^{2+} binding site. The Ca^{2+} binding site in α-lactalbumin is located in the region corresponding to the loop region 82 ~ 91 which is included in peptide fragment 59 ~ 105 in hen lysozyme that has been shown to fold in the early stage of the folding of lysozyme in the previous CHAPTER. In order to examine the effect of Ca^{2+} against the formation of region 59 ~ 105 in lysozyme, I prepared hen lysozyme possessing a Ca^{2+} binding site as in α–lactalbumin by site-directed mutagenesis (named CaB lysozyme). In CHAPTER IV, I analyzed the folding rate of CaB lysozyme from reduced form in the presence or absence of Ca^{2+} in order to examine the effect of Ca^{2+} against the early stage of lysozyme folding. As a result, it was suggested that Ca^{2+} accelerated the folding process of reduced CaB lysozyme, indicating that the formation of region connecting between α–domain and β-domain in lysozyme was participated with the early stage of lysozyme folding.

During the folding of many proteins from denatured state, aggregation frequently occurs, resulting in a decrease of the folding yield. In previous report on folding of reduced lysozyme (Goldberg *et al*, 1991), they discussed that aggregation was caused by

non-specific interaction between hydrophobic regions of polypeptide chains in an initial extremely rapid stage of the folding and that aggregation kinetically competed with folding. I found that Ca^{2+} accelerated the folding process of reduced lysozyme (CHAPTER IV). Therefore, in CHAPTER V, I examined whether aggregation would be depressed by the acceleration of the formation of the Ca^{2+} binding region, resulting in the efficient folding of reduced CaB lysozyme. As a result, I found that CaB lysozyme was efficiently folded in the presence of high concentration of Ca^{2+} and the loop region 82 ~ 91 in lysozyme formed some structure, which was different from random structure and loosely bound Ca^{2+}, in the initial extremely rapid folding stage from the reduced form.

In CHAPTER II and III, I have suggested that Trp 62 is one of the key residues *in vitro* folding of reduced lysozyme. Therefore, in CHAPTER VI, to examine whether the information of protein folding *in vivo* was similar to that of protein folding in yeast, which are usually used to the production of foreign proteins, I prepared a series of mutant lysozymes with different size of the residue at the position 62 from yeast, and analyzed the effect of the residue 62 on the correct formation of disulfide bond. By the analysis of the formation of disulfide bond, it was found that the species containing incorrect disulfide bond and free cysteine residues were present in mutant lysozymes secreted from yeast, in which the residue at the position 62 was less bulky. From these results, I suggested that the folding in the early stage of lysozyme *in vivo* was resemble to that *in vitro*.

These results were published or submitted as follow:

I; Ueda, T., Abe, Y., Ohkuri, T., Kawano, K, Terada, Y. and Imoto, T. (1995) *Biochemistry* 34, 16178-16185. (The result in CHAPTER I is partially contained in this paper)

II; Ueda, T., Ohkuri, T. and Imoto, T. (1996) *Biophys. Biochem. Res. Commun.* 228, 203-208.

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VI; Ohkuri, T., Ueda, T. and Imoto, T. (1999) submitted.

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CHAPTER I

Qualitative and Quantitative Fragmentation of Hen Lysozyme into the Peptides Containing a Single Disulfide Bond Each

ABSTRACT

I developed the method of qualitative and quantitative fragmentation of hen lysozyme into the peptides containing a single disulfide bond each. After lysozyme was cleaved by BrCN under acidic condition, the BrCN-treated lysozyme was dissolved in 0.1 M Na-K phosphate buffer (pH 6.5), and digested with the TPCK-trypsin at 40°C for 4h. These tryptic peptides were separated on RP-HPLC column, resulting in the three peptide fragments contained Cys 6 - Cys 127, Cys 30 - Cys 115, or Cys 64 - Cys 80 and Cys 94 - Cys 76. Then, the peptide fragment contained Cys 64 - Cys 80 and Cys 94 - Cys 76 was separated and lyophilized. The peptide was dissolved in 0.1 M Na-K phosphate buffer (pH 6.5). It was digested with α-chymotrypsin at 40°C for 2 h, and then with prolylendopeptidase at 40°C for 10 min. By the analysis on RP-HPLC, two disulfide bonds were divided into the peptide fragments containing a single disulfide bond each.

INTRODUCTION

The folding process of reduced protein by sulfhydryl-disulfide interchange reactions are initiated by the exchange reactions between cysteines and oxidizing reagent. As it have been shown that the folding process of a protein proceeds through a certain pathway, to analyze disulfide bond formations is one method to know the folding mechanism (Creighton, 1978). The disulfide formation pathways have been analyzed as for some proteins; BPTI (Creighton, 1978, 1990; Weissman and Kim, 1991), ribonuclease T1

(Pace and Creighton, 1986; Schonbrunner and Schmid, 1992), and hirudine (Chatrenet and Chan, 1992, 1993). Anderson and Wetlaufer (1976) analyzed the formation of disulfide bonds by means of trapping sulfhydryl-disulfide interchange reactions by alkyl reagents in the folding from reduced lysozyme. They digested the folding intermediates into peptide fragments containing disulfide bond by pepsin. However, since pepsin had low specificity, the analysis of the intermediate was not quantitative. To date, there was no method of qualitative and quantitative analysis of four disulfide bond formations in hen lysozyme. To analyze disulfide bond formations qualitatively and quantitatively, these peptides should be divided into qualitative fragmentation contained only a single disulfide bond each. Therefore, in this CHAPTER, I developed a qualitative and quantitative method to analyze four disulfide bonds in lysozyme.

MATERIALS AND METHODS

Materials

Five times recrystallized hen egg-white lysozyme was donated by QP Company (Tokyo). A column of Wakosil C18 (4.6 x 250 mm) for RP-HPLC were obtained from Wako Pure Chemical Industries, Ltd. (Osaka). TPCK-trypsin and α-chymotrypsin were the product of Worthington. All other chemicals used were of the highest quality commercially available.

BrCN treatment of lysozyme

Three milligrams of lysozyme was dissolved in 1 ml of 6 M Gdn-HCl 1 M HCl. To the solution, 150 mg of BrCN was added as a solid and stirred for 20 h at room temperature in the dark. After the evaporation of BrCN in vacuum, the residue was redissolved in 1 ml of 10% aqueous acetic acid and dialyzed against the same solution. The solution was lyophilization.

Tryptic digestion

Two milligrams of BrCN-treated lysozyme was dissolved in both 500 μl of H_2O and 500 μl of 0.2 M Na-K phosphate buffer (pH 6.5). To the solution, 50 μg of TPCK-trypsin was added and incubated at 40 °C for 4 h.

Digestion of peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 by α-chymotrypsin and prolylendopeptidase

Ten nanomoles of peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 separated on RP-HPLC was dissolved in 500 μl of 0.1 M Na-K phosphate buffer (pH 6.5). It was digested with two milligrams of α-chymotrypsin was added to the solution and incubated at 40°C for 2 h, and then 0.01 unit of prolylendopeptidase was added and incubated at 40°C for 10 min.

Analysis

The tryptic peptides derived from the BrCN-treated lysozyme were applied to the column of Wakosil C18 equipped to HPLC. The column was eluted with a gradient of 50 ml of 1% acetonitrile and 50 ml of 50% acetonitrile both containing 0.1% HCl at a flow rate of 0.6 ml/min. The digests of tryptic peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 derived with α-chymotrypsin and prolylendopeptidase were applied to the column of Wakosil C18 equipped to HPLC. 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. Performic acid oxidation of the peptides was carried out according to the method of Harris (Harris, 1967). Amino acid analysis was performed on a Hitachi L-8500 amino acid analyzer after hydrolysis of a sample in 6 M HCl at 110°C for 20 h.

RESULTS

Digestion of BrCN-treated lysozyme with TPCK-trypsin

The intact lysozyme is hardly digested by TPCK-trypsin because it is higher stability. In order to decrease the stability of lysozyme, the peptide bond at Met 12 and Met 105 in lysozyme was cleaved by BrCN (see Figure I-1). Then, BrCN-treated lysozyme was digested by TPCK-tripsin at pH 6.5 and was applied to the column of RP-HPLC. The pattern was shown in Figure I-2. From amino acid analysis after acid hydrolysis (data not shown), the peak-a corresponded to the peptides Cys 6 ~ Homoserine 12 plus Gly 126 ~ Leu 129 which contained a disulfide bond, Cys 6 - Cys 127, the peak-b to the peptides Gly 22 ~ Lys 33 plus Cys 115 ~ Arg 121 which contained the disulfide bond, Cys 30 - Cys 115, and the peak-c corresponded to the peptides Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 which contained two disulfide bonds, Cys 64 - Cys 80 and Cys 76 - Cys 94. The peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 was lyophilized for further digestion.

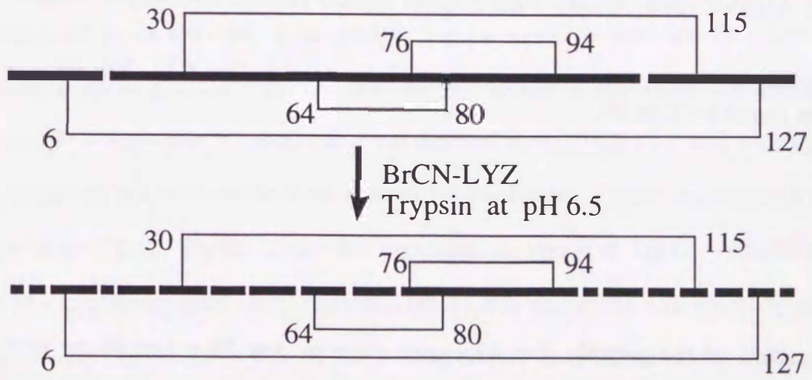

Figure I-1 Tryptic peptides derived from BrCN-treated lysozyme.

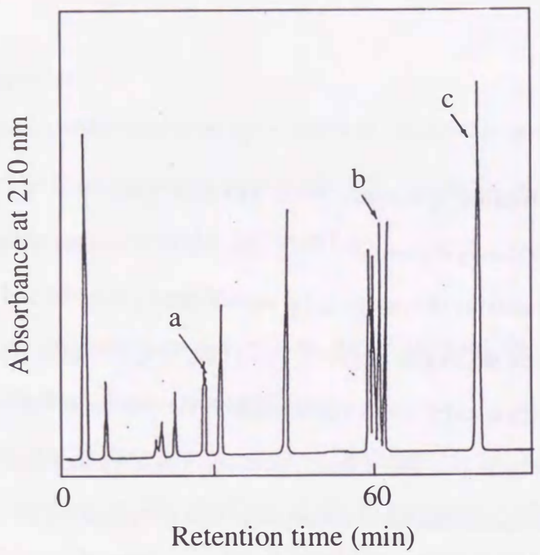

Figure I-2 RP-HPLC of tryptic peptides derived from BrCN-treated lysozyme. The column (Wakosil C18, 4.6 mm x 250 mm) was eluted with a gradient of 50 ml of 1% acetonitrile and 50 ml of 50% acetonitrile both containing 0.1% HCl at a flow rate of 0.6 ml/min.

Arg68 Gly67 Asp66 Asn65 Cys64 Trp63 Trp62

Asn74 Leu75 Cys76 Asn77 Ile88 Pro79 Cys80 Ser81 Ala82 Leu83 Leu84 Ser85

Lys96 Ala95 Cys94 Asn93 Val92 Ser91 Ala90 Thr89 Ile88 Asp87 Ser86

Figure I-3 The amino acid sequence of the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96.

Digestion of the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 by both α-chymotrypsin and prolylendopeptidase

The amino acid sequence of the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 is shown in Figure I-3. To divide the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 contained two

disulfide bonds, Cys 64 - Cys 80 and Cys 94 - Cys 76, into respective peptide contained a single disulfide bond, the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 must be specifically cleaved between Cys 76 ~ Cys 80 and Cys 80 ~ Cys 94.

Alpha-chymotrypsin was a serine protease with specificity for cleaving the C-terminal of aromatic or hydrophobic residue. In our laboratory, it was found that α-chymotrypsin digested at a few position between Cys 80 and Cys 94 in the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 (Aoki, 1988). On the other hand, prolylendpeptidase is a serine protease with specificity for cleaving the Pro-X bond. (Yoshimoto, *et al.* 1978; 1980). A proline is located at the position 79 between Cys 76 and Cys 80. Therefore, I tried to digest the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 with α-chymotrypsin and prolylendpeptidase. The peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 was dissolved in 0.1 M Na-K phosphate buffer (pH 6.5). It was digested with α-chymotrypsin at 40°C for 2 h, and then with 0.01 unit of prolylendopeptidase at 40°C for 10 min, and was applied to column of RP-HPLC. The elution pattern of RP-HPLC was shown in Figure I-4. In order to examine the amino acid composition, each peptides was isolated, and each amino acid analysis after performic acid oxidation was conducted (Table I-1). The assignment of each peptide in Figure I-4 was shown in Figure I-5 by enclosing each with a dotted line on the sequence of the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96. Because peptides g and h could be detected at 280 nm, it was suggested that they included Trp. However, no amino acid was detected in peptide h after acid hydrolysis. Trp was eluted between peptide d and e under the conditions. Therefore, peptide h was identified as Trp 62 - Trp 63. Under this condition, the region of Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 was specifically cleaved at Pro 79, Ala 82, Leu 83, and Val 92 by these enzymes. By this procedure (Figure I-6), I could divide lysozyme into peptide fragments containing a single disulfide bond each qualitatively and quantitatively.

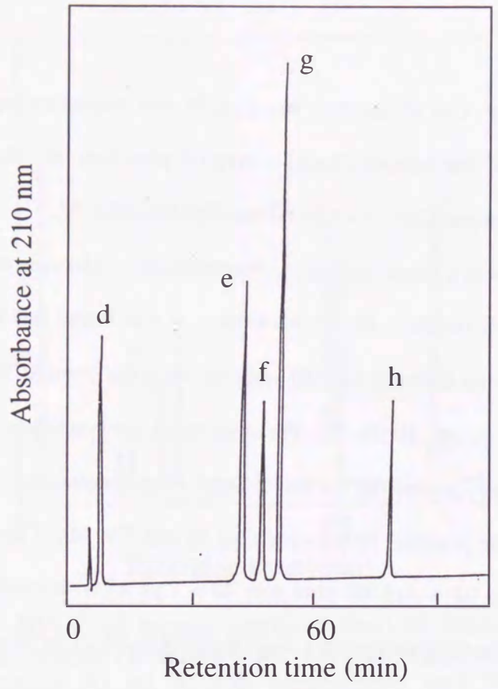

Figure I-4 RP-HPLC of protease digest of the peptide corresponded to Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 with α-chymotrypsin and prolylendopeptidase. The column (Wakosil C18, 4.6 mm x 250 mm) was eluted with a gradient of 40 ml of 1% acetonitrile and 40 ml of 40 % acetonitrile both containing 0.1% HCl at a flow rate of 0.6 ml/min.

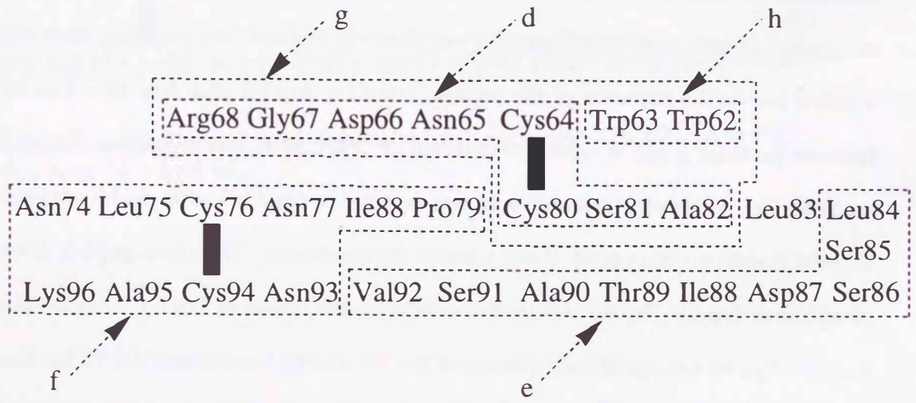

Figure I-5 The amino acid sequence of peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96. Thick lines indicate the disulfide bond. The assignment of each peptide in Figure I-4 was carried out and shown by enclosing with a dotted line.

Table I-1 Amino acid composition of the amino acid after performic acid oxidation in Figure I-4.

Amino acid	peak d		peak e		peak f		peak g		peak h	
	Calc.	Theory	Calc.	Theory	Calc.	Theory	Calc.	Theory	Calc.	Theory
Asp	1.7	2	2.0	2	3.0	3	2.1	2	0	0
Thr	0	0	0.8	1	0	0	0	0	0	0
Ser	1.1	1	2.7	3	0	0	0.8	1	0	0
Glu	0	0	0	0	0	0	0	0	0	0
Gly	1.3	1	0	0	0	0	1.2	1	0	0
Ala	1.0	1	1.0	1	1.0	1	1.0	0	0	0
Val	0	0	1.0	1	0	0	0	0	0	0
Met	0	0	0	0	0	0	0	0	0	0
Ile	0	0	0.9	1	0.7	1	0	0	0	0
Leu	0	0	1.0	1	0.9	1	0	0	0	0
Tyr	0	0	0	0	0	0	0	0	0	0
Phe	0	0	0	0	0	0	0	0	0	0
Lys	0	0	0	0	0.9	1	0	0	0	0
His	0	0	0	0	0	0	0	0	0	0
Arg	0.7	1	0	0	0	0	0.8	1	0	0
Pro	0	0	0	0	1.0	1	0	0	0	0
Cys-SO_3H	2.0	2	0	0	2.0	2	2.0	0	0	0
Trp	-	-	-	-	-	-	+	+	+	+
Assigned residue number	64-68 80-82		84-92		74-79 96-93		62-64-68 80-82		62-63	

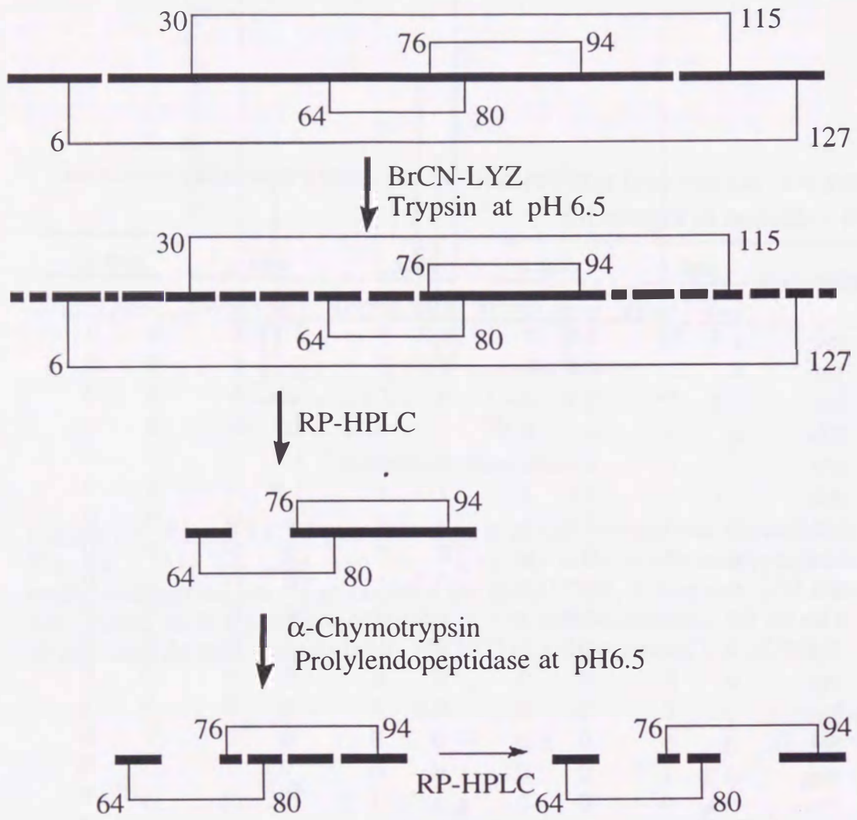

Figure I-6 Method of the division of all four disulfides in hen lysozyme.

DISCUSSION

The study of the folding mechanisms by Creighton (1988) was based on the strategy that the disulfide bonding patterns of intermediates trapped at various times during folding provide a probe for elucidating transient structural features. In order to isolate

and structurally characterize the intermediate in oxidative folding, it is necessary to stop thiol-disulfide exchange reactions. As the pK_a of the thiol is 8.5 and the sulfhydryl-disulfide interchange reaction proceeds at the dissociated, the analysis of disulfide bond formation must be carried out at acidic condition. I developed the method in which the four disulfide bonds were qualitatively and quantitatively divided into peptides containing a single disulfide bond with digestion with trypsin, α-chymotrypsin, and prolylendopeptidase at pH 6.5. By using this method, it would be possible to examine the formation of the disulfide bonding in oxidative folding of lysozyme. I examined the formation of disulfide bond of lysozyme by using this method in following chapter.

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CHAPTER II

Identification of the Peptide Region that Folds Native Conformation in the Early Stage of the Folding of Reduced Lysozyme

ABSTRACT

I prepared three peptide fragments (fg. 59 ~ 105, fg. 63 ~ 105 and fg. 64 ~ 105) by the BrCN-treatment of mutant lysozymes where Ile 58, Trp 62 and Trp 63 were mutated to Met, respectively. From the analysis of formation of the disulfide bonds among Cys 64, Cys 76, Cys 80 and Cys 94 in the oxidation of each peptide fragment from the reduced form, Trp 62 and Trp 63 were required for the effective formation of two disulfide bonds. Especially, Trp 62 was found to be involved in the correct formation of the disulfide bonds.

INTRODUCTION

Kim and Baldwin (1982) 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 kinetic analysis for the early stage of the protein folding by means of CD or NMR spectroscopy with pulse-labeling technique would give us many information on the mechanism of protein folding (Udgaonkar and Baldwin, 1988; Roder *et al*, 1992; Radford *et al*, 1992). On the other hand, to dissect protein is a method to know how some parts in the whole protein contribute to 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.

On four disulfide bonds Cys 6 - Cys 127, Cys 30 - Cys 115, Cys 64 - Cys 80 and Cys 76 - Cys 94 in lysozyme, when reduced lysozyme was folded by sulfhydryl-disulfide interchange reactions, two disulfide bonds Cys 64 - Cys 80 and Cys 76 - Cys 94 which are located inside of the molecule, formed earlier than the other ones (Anderson and Wetlaufer, 1976). Therefore, it should be reasonable to focus my attention on the peptide region around these two cystines. It was reported that the peptide fragment between Lys 13 and Homoserine 105 (fg. 13 ~ 105) could fold to the peptide structure that has the affinity to lysozyme substrate-immobilized column (Johnson *et al*, 1978). However, there was no experimental data for the oxidation of the shorter peptide than the fg. 13 ~ 105 from the reduced form. Therefore, to identify the region formed in the early stage of the protein folding, I tried to analyze the minimum region required for the formation of these two disulfide bonds by using peptide fragment. As a result, the peptide fragment 59 ~ 105 of hen lysozyme formed the correct disulfide bond from reduced form without the help of the other peptide region.

MATERIALS AND METHODS

Materials

Sephadex G-75 was obtained from Pharmacia. A column of Cosmosil C4 (4.6 x 150 mm) were obtained Nacalai Tesque Inc. (Kyoto). A column of Wakosil C18, TPCK-trypsin, α-chymotrypsin, and prolylendpeptidase were purchased as shown in the previous CHAPTER. All other chemicals used were of the highest quality commercially available.

Expression and purification of mutant lysozymes

I prepared mutant lysozymes where Ile 58, Trp 62 and Trp 63 in hen lysozyme were mutated to Met, respectively, by site-directed mutagenesis according to the methods of

Hashimoto *et al.* (1996). These mutations in the lysozyme gene were confirmed by DNA sequencing. These yeast *Saccharomyces cerevisiae* AH22 transformants prepared were cultivated at 30°C for 125 h to express and secrete the mutant lysozyme from yeast as described in the literature (Hashimoto *et al.* 1996). The purification of mutant lysozymes were carried out according to the previous report (Ueda *et al*, 1994).

Preparation of peptide fragments

Three peptide fragments (fg. 59 ~ 105, fg. 63 ~ 105, and fg. 64 ~ 105) were prepared by BrCN-treatment of mutant lysozyme (I 58 M lysozyme, W 62 M lysozyme, and W 63 M lysozyme), respectively. Five milligrams of mutant lysozymes were dissolved in 1 ml of 6 M Gdn-HCl containing 1M HCl, respectively. To each solution, 100 mg of BrCN was added and stirred for 20 h 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 x 90 cm). Each desired peptide (fg. 59 ~ 105, fg. 63 ~ 105, and fg. 64 ~ 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.

Oxidation of the reduced peptide fragments and analysis of the formation of disulfide bonds

Reduction and oxidation of reduced peptide fragments at a concentration of 0.3 μM was carried out in 0.2 M Tris-acetate buffer (pH 8.0) at 37°C according to the previous paper (Ueda *et al*, 1990) with a slight modification. Fifty-eight nanomoles of peptide fragments were dissolved in 2 ml of 8 M urea solution ((0.584 M Tris-HCl buffer (pH 8.6) containing 5.37 mM EDTA)). The solution was added 25 μl of 2-mercaptoethanol and was incubated at 40°C for 90 min (reduced solution). On the other hand, 198 ml of

renaturation buffer (0.2 M Tris-acetate buffer containing 81 mg GSSG) was preincubated for 5 min at 40°C. Oxidation of the reduced fragmentation was initiated by adding 2 ml of the reduction solution to 198 ml of the renaturation buffer with stirring the reaction of each oxidized peptide fragments with *N* -ethylmaleimide (10 mM) at pH 5.5, the pH of each reaction mixture were lowered to 3.0 and dialyzed against 10% aqueous acetic acid using dialyzing tube (cutoff 3,500). For further purification, each dialysates were applied to the column of Cosmosil C4 (4.6 x 150 mm) equipped with HPLC that were equilibrated with 1 % acetonitrile containing 1 % acetic acid at a flow rate of 1 ml/min. The column was eluted with 50 % acetonitrlile containing 1 % acetic acid. The eluted peptide fragments fraction were lyophilized. Analysis of the disulfide bond of each oxidized peptide fragment was carried out using two step protease digestion according to the method of CHAPTER I.

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 2 h.

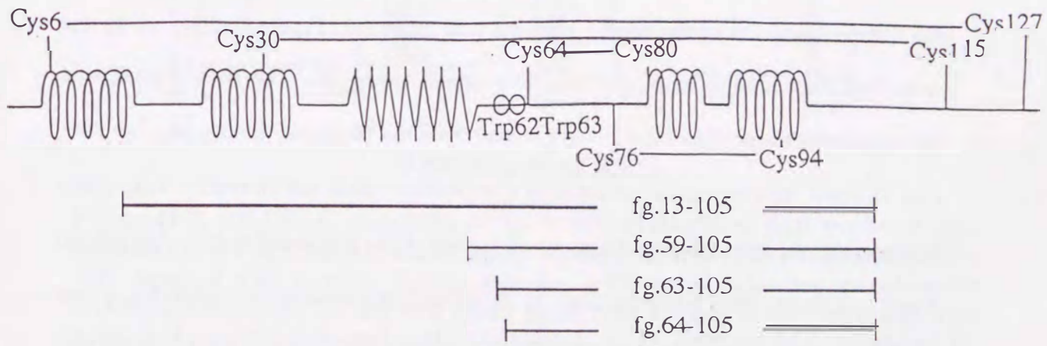

Figure II-1 Secondary structures and the positions of cystines in hen lysozyme.

RESULTS AND DISCUSSION

In the fg. 13 ~ 105 (Figure II-1), there were two helical structures (24 ~ 36 and 88 ~ 98), one β-sheet (44 ~ 60) , two cystines (Cys 64 - Cys 80, Cys 76 - Cys 94) and one half cysteine (Cys 30). The synthetic peptide (41 ~ 60) including the β-sheet was apt to oligomerize (Yang *et al*, 1994). On the other hand, the modification of Trp 62 affected the folding of reduced lysozyme (Ueda *et al*, 1990; Abe, 1995). Therefore, I prepared three peptide fragments, fg. 59 ~ 105, fg. 63 ~ 105, and fg. 64 ~ 105. In order to examine whether each oxidized fragment can fold to intact one, I analyzed the formation of disulfide bond among four cysteines in each oxidized fragment. The analysis of disulfide bonds among Cys 64, Cys 76, Cys 80 and Cys 94 was carried out in two steps according to the method of CHAPTER I. Figure II-2A shows RP-HPLC pattern of the peptide derived from tryptic digestion of the oxidized peptide fragment 59 ~ 105. The elution position of peak-a was identical to that of the peptides Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 as was shown in CHAPTER I. Moreover, the amino acid composition of peak-a was the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 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 reduced products of the tryptic peptides was analyzed (Figure II-2B). After the reduction, peak-a disappeared and two new peaks appeared. The former peak was identified to be the peptide Trp 62 ~ Arg 68 and the latter one to be the peptide Asn 74 ~ Lys 96 from the respective amino acid composition (data not shown). The result indicates that the original peak-a was not a complex of the peptides Trp 62 ~ Arg 68 and Asn 74 ~ Lys 96. This was supported by the result that peak-a did not include any free cysteine since there was no carboxymethylated cysteine in the sample where peak-a was incubated with monoiodoacetic acid in 8.0 M urea solution (data not shown).

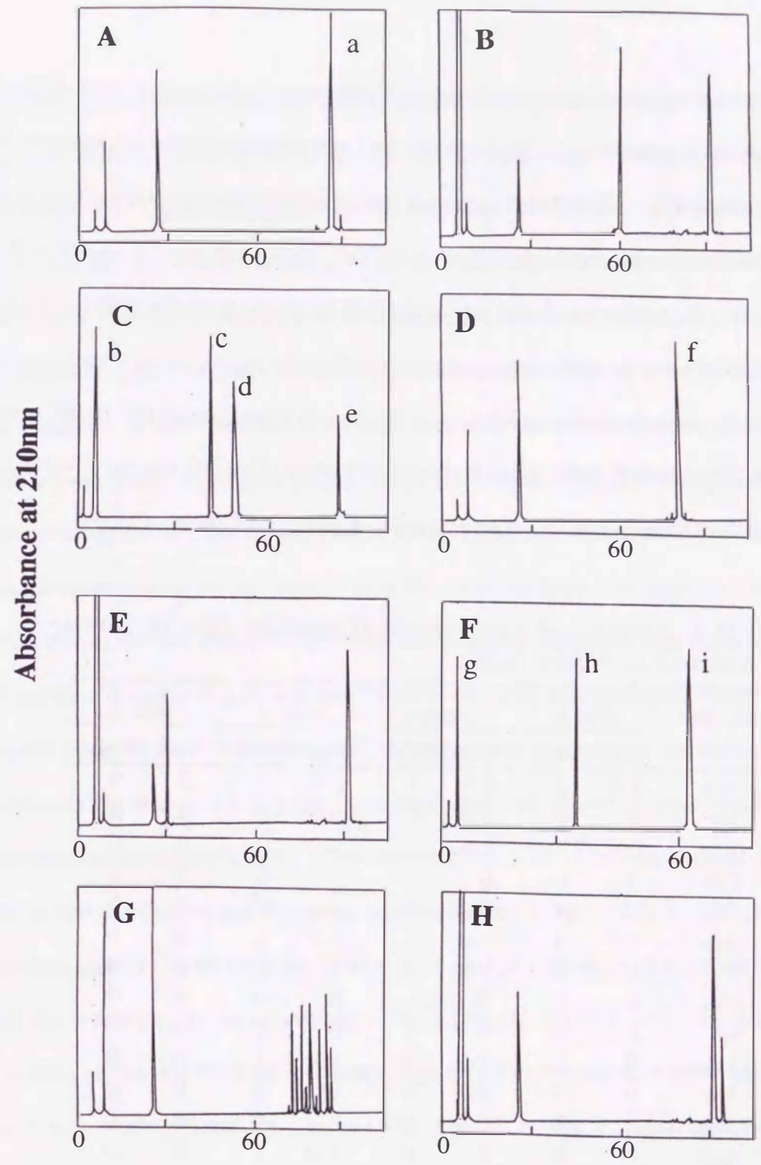

Figure II-2 RP-HPLC of digests of the oxidized fragment with protease on a column (Wakosil C18, 4.6 x 250 mm). A, derived from the oxidized fg. 59 ~ 105 digested with trypsin; B, the peptides in Figure II-2A after reduction by 2-mercaptoethanol; C, derived from peak a in Figure II-2A digested with both α-chymotorypsin and prolylendpeptidase; D, derived from oxidized fg. 63 ~ 105 digested with trypsin; E, the peptides in Figure II-2D after reduction by 2-mercaptoethanol; F, derived from peak-f in Figure II-2D digested with both α-chymotorypsin and prolylendpeptidase; G, derived from oxidized fg. 64 ~ 105 digested with trypsin; H, the peptides in Figure II-2G after reduction by 2-mercaptoethanol.

For further investigation of disulfide bonds in the peak-a, the peptide was isolated, lyophilized, and digested with α-chymotrypsin and prolylendopeptidase according to the method of CHAPTER I. RP-HPLC pattern of the digested peptide is shown in Figure II-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 composition after the peroxidation of each peptide was analyzed. As was shown in Table II-1, the correct formation of two cystines was confirmed. Therefore, β-sheet region and one half cystine (Cys 30) was not involved in the correct formation of disulfide bonds (Cys 64 - Cys 80 and Cys 76 - Cys 94).

Table II-1 Amino acid composition of peptides after performic acid oxdation in Figure II-2C.

Amino acid	peak b		peak c		peak d		peak e	
	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	

Similarly, Figure II-2D shows RP-HPLC pattern of the peptides derived from the tryptic digestion of the oxidized fg. 63 ~ 105. The amino acid composition indicated that peak-f was the peptide Trp 63 ~ Arg 68 plus Asn 74 ~ Lys 96 where these peptides covalently connected through disulfide bonds (data not shown). This is consistent with the observation that peak-f eluted earlier than the peak-a due to the truncation of Trp 62 from the peptide Trp 62 ~ Arg 68. Moreover, in order to neglect the possibility that peak-f is not a complex of these peptides, RP-HPLC pattern of the reduced products of the tryptic peptides was analyzed (Figure II-2E). After the reduction, peak-f disappeared and two new peaks appeared. The former peak was identified to be the peptide Trp 63 ~ Arg 68 and the latter one to be the peptide Asn 74 ~ Lys 96 from the respective amino acid composition (data not shown). The result indicates that the peak-f was not a complex of the peptides Trp 63 ~ Arg 68 and Asn 74 ~ Lys 96. This was supported by the result that original peak-f did not include any free cysteine since there was no carboxymethylated cysteine in the sample where peak-f was incubated with monoiodoacetic acid in 8.0 M urea solution (data not shown). Then, the analysis of the disulfide bond of the peak-f was carried out by protease digestion described above (Figure II-2F). The presence of Trp was examined by monitoring the elution of each peptide at 280 nm. From amino acid composition after the peroxidation of each peptide (Table II-2), the predominant formation of non-native disulfide bonds (Cys 64 - Cys 76 and Cys 80 - Cys 94) was found to occur. Therefore, the peptide Asn 59 ~ Trp 62 was concluded to be involvement in the correct formation of disulfide bonds. In the previous data, it was suggested that the interaction between the hydrophobic side of the helix and Trp 62 and/or Trp 63 were important in the early stage of the folding of reduced lysozyme (Ueda *et al*, 1994). In the folded state, Trp 63 is closely located near the α-helix 88 ~ 98 but Trp 62 is not (Ueda *et al*, 1994). However, since there is generally the interactions between the amino acid side chains in the sequence of Trp-Trp or Trp-Tyr (Rizzo *et al*, 1983), the interaction between Trp 62 and Trp 63 must be present in the reduced form.

Namely, the orientation of Trp 63 can be restricted by the presence of Trp 62 even in the reduced form. The interaction between them would lead these cystines (Cys 64, Cys 76, Cys 80 and Cys 94) to the formation of correct disulfide bonds (Cys 64 - Cys 80 and Cys 76 - Cys 94) in the fg. 59 ~ 105. On the other hand, in the fg. 63 ~ 105, as the residues Asn 59 ~ Trp 62 (especially Trp 62) were truncated from the fragment, Trp 63, which is not restricted, may freely interact with the hydrophobic side of helix 88 ~ 98. As the result, the non-native disulfide bonds (Cys 64 - Cys 76 and Cys 80 - Cys 94) formed predominantly.

Table II-2 Amino acid composition of peptides after performic acid oxidation in Figure II-2F.

Amino acid	peak g		peak h		peak i	
	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	

Figure II-2G shows RP-HPLC pattern of the peptide derived from tryptic digestion of the oxidized fg. 63 ~ 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 peptide is shown in Figure II-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 Asn 74 ~ Lys 96 while the minor peak was one reacted with *N*-ethylmaleimide which was employed for the trapping of the oxidized peptides. Therefore, the complicated pattern in the latter part on RP-HPLC in Figure II-2H was majorly due to the formation of the scrambled disulfide bonds, which somewhat contains incomplete disulfide bonds. Namely, further truncation of Trp 63 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 Trp 62 and/or Trp 63 interacted with the α-helix 88 ~ 98 in the early stage of the folding of reduced lysozyme (Ueda *et al*, 1994).

The results are summarized as follows 1) I identified the peptide region 59 ~ 105 to lead four cystines, which forms in the early stage of the folding of reduced form, to form native disulfide bonds, 2) Trp 62 may be involved in the correct formation of these disulfide bonds from reduced form, 3) The interaction between Trp 62 and/or Trp 63 and the α-helix 88 ~ 98 (Ueda *et al*, 1994) was confirmed to be essential in the effective formation of these disulfide bonds from reduced form. The identification of the peptide region folds in the early stage of the folding 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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CHAPTER III

Involvement of the Peptide Region 59 ~ 105 in Hen Lysozyme in the Formation of Active Structure in Folding Process

ABSTRACT

As shown in CHAPTER II, the peptide fragment 59 ~ 105 of hen lysozyme formed the correct disulfide bonds from reduced form without the help of the other peptide region. In order to investigate whether the formation of the peptide fragment 59 ~ 105 is involved in the formation of active structure in folding process, I carried out association experiments between the peptide fragment 59 ~ 105 and the residual peptide region of hen lysozyme (fg. 1 ~ 58 covalenty connected to fg. 106 ~ 129 by disulfide bonds). From lytic activity and the analysis of ion-exchange HPLC, these peptides were found to form a native like molecule. On the other hand, the peptide fragments 59 ~ 105 from the mutant where Cys 64, Cys 80, Cys 76, and Cys 94 are mutated to Ala or where Trp 62 is mutated to Gly could not form the native like molecule with the same residual peptide fragment. Based on the information, I designed the mutant lysozyme where Cys 6, Cys 30, Cys 115, and Cys 127 are simultaneously mutated to Ala and folded it from the reduced form. From lytic activity, peptide analysis, circular dichroism spectra, Gdn-HCl denaturation, and fluorescent spectra in the presence of 1-anilinonaphthalene-8-sulfonic acid, the folded mutant lysozyme formed correct disulfide bonds and some tertiary structure, and showed the activity. From above results, it was concluded that the formation of peptide region 59 ~ 105 in lysozyme was cue in the folding process leading to active structure of the reduced lysozyme.

INTRODUCTION

In CHAPTER II, from the analysis using peptide fragments, the reduced peptide fragments 59 ~ 105 was shown to oxidize to the intact fragment quantitatively judging from the formation of two cystines Cys 64 - Cys 80 and Cys 76 - Cys 94 . This result indicates that the peptide region 59 ~ 105 folds without help of the other region in the folding of the reduced lysozyme. However, I do not refer to whether the formation of the peptide region 59 ~ 105 is involved in the preceding folding of native structure required for manifestation of lysozyme activity. In this CHAPTER, using peptide fragments and mutant lysozyme, I elucidated that the formation of the peptide region 59 ~ 105 was closely involved in the preceding folding process leading to active structure of the reduced lysozyme.

MATERIALS AND METHODS

Materials

Hen egg-white lysozyme, TPCK-trypsin, α–chymotrypsin, prolylendopeptidase, a column of Wakosil C18 (4.6 x 450 mm) were obtained as shown in the previous CHAPTER. A column of Asahipak ES-502C and TSK-gel G3000SW were obtained from Asahi Chemical Industry Co. (Tokyo) and Tosoh Co. (Tokyo), respectively. Cosmosil C18 was purchased from Nakarai Tesque (Kyoto). 1-Anilinonaphthalene-8-sulfonic acid was purchased from Molecular Probes, Inc. *Micrococcus luteus* was purchased from Sigma. All other chemicals used were of the highest quality commercially available.

Site-directed mutagenesis of hen lysozyme

Site-directed mutagenesis of hen lysozymes (I 58 M lysozyme, W 62 G I 58 M lysozyme, C 64 A C 76 A C 80 A C 94 A I 58 M lysozyme, and C 6 A C 30 A C 115 A

C 127 A lysozyme) were performed as shown in CHAPTER II. The mutation in the lysozyme gene were confirmed using a DNA sequence analysis.

Expression and purification of mutant lysozymes

Expression from yeast (*Saccharomyces cerevisiae*), and purification of I 58 M lysozyme and W 62 G I 58 M lysozyme were performed as shown in CHAPTER II. Expression of Ser^{-1} C 64 A C 76 A C 80 A C 94 A I 58 M lysozyme and Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme were performed with an expression vector pET22b (+) in *E.coli* BL21 (DE3) cells according to the previous paper (Mine *et al*, 1997). The strain was grown at 37°C in LB medium. When the absorbance of the culture medium at 660 nm reached 0.9, hen lysozyme gene was induced for 2 h after the addition of IPTG to a final concentration of 1.0 M. The purification of Ser^{-1} mutant lysozymes produced in *E. coli* was carried out according to the method of Mine *et al* (1997). Cells were harvested by centrifugation for 10 min at 8000 r.p.m. Pellets were suspended in 30 ml of 0.05 M 3-(*N*-morpholino)-2-hydroxypropaneesulfonic acid (MOPSO), pH 7.0 and 0.2 mM 4-(2-aminoethyl) fluoride hydro-chloride (Perfabloc SC) and then sonicated for 30 s 20 times in an ice-water bath. The mixture was centrifuged for 30 min at 7000 r.p.m. The precipitates were suspended in 6.0 M Gdn-HCl solution ((0.575 Tris-HCl buffer containing 6.0 M Gdn-HCl and 5.25 mM EDTA (pH 8.6))), degassed by aspiration, purged with nitrogen and then reduced with 50 μl of mercaptoethanol at 40°C for 1 h. The reduced mixture was diluted into 100 ml of 10% acetic acid with dropwise addition and vigorous stirring in order to extract the reduced mutant lysozyme. The supernatants were lyophilized and then chromatographed on a column of Sephadex G-75 (1.5 cm x 150 cm) with 10% acetic acid as eluent. The fractions of the main peak were collected and lyophilized to give fairly pure lysozyme derivative in an inactive form.

Preparation of peptide fragments

Each desired peptide (fg. 1 ~ 58 + fg. 106 ~ 129, fg. 59 ~ 105, C 64 A C 76 A C 80 A C 94 A fg. 59 ~ 105, and W 62 G fg. 59 ~ 105) was prepared according to the method of CHAPTER II.

Association of the peptide fragment 59 ~ 105 or the mutant peptides (C 64 A C 76 A C 80 A C 94 A fg. 59 ~ 105 and W 62 G fg. 59 ~ 105) with the residual peptide fragment (fg. 1 ~ 58 + fg. 106 ~ 129)

The equimolar mixture of the lyophilized peptide fragment 59 ~ 105 or mutant peptides (C 64 A C 76 A C 80 A C 94 A fg. 59 ~ 105 and W 62 G fg. 59 ~ 105) and the lyophilized residual peptide fragment 1 ~ 58 + 106 ~ 129 of hen lysozyme were dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 6.0 M Gdn-HCl and incubated at 40°C for 30 min under the non-reducing condition in the concentration of 12.5 - 200 μg/ml as lysozyme molecule. Then, the denatured fragments solution was slowly and exhaustively dialyzed against 0.1 M Tris buffer (pH 8.0) containing 1.0 M urea or 0.1 M Tris buffer (pH 8.0) and 4°C.

Ion-exchange HPLC of the mixture of peptide fragments

Five hundred microliter of the above dialysate was applied to the ion-exchange column (Asahipak ES-502C, 7.6 x 100 mm) equipped to HPLC. The column was eluted with a gradient of 40 ml of 0.1 M sodium acetate buffer (pH 5.0) and 40 ml 0.1 M sodium acetate buffer (pH 5.0) containing 1.0 M NaCl and a flow rate of 1.0 ml/min.

The lytic activity

The lytic activities of the mixture of the peptide fragments or Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme were measured turbidimetrically at 450 nm, at pH 7.0 and 30°C. To 2 ml suspension of *Micrococcus luteus* in 0.05 M Na-K phosphate buffer (pH 7.0)

was added 100 μl of lysozyme solution and then the turbidity decrease was monitored at 450 nm.

The renaturation of Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme

The renaturation of Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme derived from the inclusion body was performed according to the methods of Maeda *et al* (1995) as follows with a slight modification. Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme (500 μg) was dissolved in 5 ml of 8.0 M urea solution ((0.584 M Tris-HCl (pH 8.0) containing 5.37 mM EDTA and 8.125 M urea)). The solution was added 25 μl of 2-mercaptoethanol, and incubated at 40°C for 1h for reduction. To the reduced solution, 81 mg of GSSG was added. The redox solution was dialyzed against 200 ml of the renaturation buffer ((0.1 M Tris-HCl (pH 8.0))) containing 8.0 M urea, 162 mg of GSSG and 50 μl of 2-mercaptoethanol with stirring. The urea concentration of dialyzing bottle was gradually diluted with 1000 ml of the renaturation buffer containing 81 mg of GSSG and 25 μl of 2-mercaptoethanol at a flow rate of 0.1 ml/min by a high pressure pump.

The purification of renatured Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme

After the renaturation of Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme, the pH of the supernatant of dialysate was lowered to 5.5 by the addition of acetic acid and 62.5 mg of *N*-ethylmaleimide was added to the dialysate. After the solution was stirred for 30 min at room temperature, the pH of the solution was lowered to 3.0 by adding acetic acid to stop the reaction. The solution was applied directly to the reversed-phase column (Cosmosil C18, 1.5 mm x 150 mm) which was equilibrated with 10% acetonitrile containing 0.1% HCl. The column was eluted with 60% acetonitrile containing 0.1% HCl.

Analysis of disulfide bond in Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme

The analysis of disulfide bond in the purified Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme was performed according to the method of CHAPTER I.

Denaturation Experiments

Denaturation experiments of Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme against Gdn-HCl in 0.1M sodium acetate buffer (pH 5.5) and 35°C was carried out by monitoring fluorescence at 360 nm (excitation at 280 nm).

RESULTS AND DISCUSSION

Association of the peptide fragment 59 ~ 105 with the residual peptide fragment (1 ~ 58 + 106 ~ 129) in wild-type lysozyme

In order to investigate whether the peptide fragment 59 ~ 105, which could fold without help of the other peptide fragment of hen lysozyme, is involved in the preceding folding process required for active structure of reduced lysozyme, I carried out the association experiments between the peptide fragments in hen lysozyme. Under non-reducing condition, the equimolar mixture of the lyophilized peptide fragment 59 ~ 105 and the lyophilized residual peptide fragment of hen lysozyme (the peptide fragments 1 ~ 58 and 106 ~ 129 where these peptide fragments are connected by two disulfide bonds, Cys 6 - Cys 127 and Cys 30 - Cys 115) were dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 6.0 M Gdn-HCl and incubated at 40°C and 30 min at the concentration of 50 μg/ml as lysozyme molecule. Then, the solution containing denatured fragments was slowly and exhaustively dialyzed against 0.1 M Tris-HCl buffer containing 1.0 M urea (pH 8.0) and 4°C. A slight lytic activity against *Micrococcus luteus* was observed for the

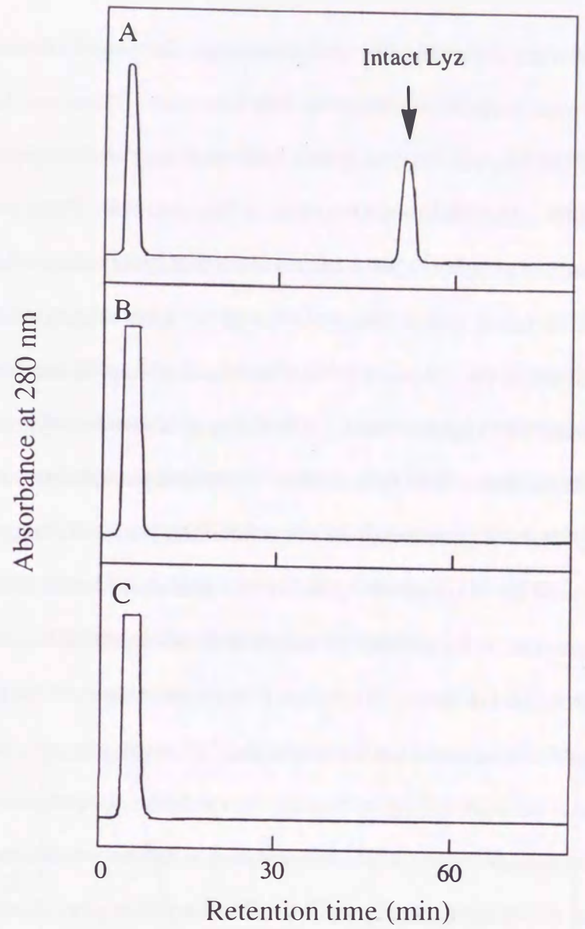

Figure III-1 Ion exchange chromatography of the mixture of peptide fragments. A, fg. 59 ~ 105 and fg. 1 ~ 58 + 106 ~129; B, 4(CA) fg. 59 ~ 105 and fg. 1 ~ 58 + 106 ~ 129; C, W 62 G fg. 59 ~ 105 and fg. 1 ~ 58 + 106 ~ 129. The column (Asahi-pak) was eluted with a gradient of 40 ml of 0.1 M sodium acetate buffer (pH 5.0) and 40 ml 0.1 M sodium acetate buffer containing 1 M NaCl (pH 5.0) at a flow rate of 1.0 ml/ min.

dialysate, indicating the formation of structure acquired for activity. To confirm the structure, the dialysate was applied to the ion-exchange HPLC column (Figure III-1A). The arrow indicates the retention time of intact lysozyme. Only one peak was eluted at the same position with intact lysozyme. Since the net charge in hen lysozyme under the

HPLC condition did not change by BrCN cleavage, the result indicated that a part of associated peptide fragments formed tertiary structure. These results mean that the association of the peptide fragment 59 ~ 105 with the residual peptide fragment of lysozyme results in the formation of the native like molecule. From comparison of the area of the resulting peak with that of known amount of lysozyme, the formation yield of the native like molecule was determined to be 23%. Lytic activity of nicked lysozyme whose peptide bonds were cleaved at the C-terminus of Met 12 and Met 105 by BrCN against *M. luteus* was approximately 10% of that of native lysozyme (Bonavida *et al*, 1969). The considerably low lytic activity of the dialysate derived from the mixture between the peptide fragments may be reasonable because the formation yield was low and the lytic activity of the resulting native like molecule would be lower than BrCN nicked lysozyme due to the presence of further nick on the peptide bond at position 58.

The formation yield of native like molecule from the mixture of the peptide fragments was not so good. I examined the formation yield of native like molecule in the various concentration of the peptide fragments under the condition of equimolar mixture between the lyophilized peptide fragment 59 ~ 105 and the lyophilized residual peptide fragment of hen lysozyme (Open circles in Figure III-2). The formation yield of native like molecule below the concentration of 50 μg/ml increased with an increase of the concentration because its formation the association between these peptide fragments. However, the formation yield over concentration of 50 μg/ml decreased with an increase of the concentration. I thought this decrease to be the result of the aggregation caused by the unfavorable interactions between the peptide fragments. In order to elucidate the above consideration, the equimolar mixture of the lyophilized peptide fragment 59 ~ 105 and the lyophilized residual peptide fragment of lysozyme dissolved in 0.1 M Tris-HCl buffer (pH 8.0) containing 6.0 M Gdn-HCl at various concentrations under non-reducing condition was slowly and exhaustively dialyzed against 0.1 M Tris-HCl buffer (pH 8.0) and 4°C without urea. The formation yield of native like molecule are shown in Figure

III-2 (Closed circles). The formation yield of native like molecule at a concentration of 25 μg/ml in the absence of urea was higher than that in the presence of 1.0 M urea because the association between the peptide fragments might be favorable in the absence of urea. However, the formation yield over concentration of 50 μg/ml decreased with an increase of the concentration, indicating the increase of aggregation caused by the unfavorable interactions between the peptide fragments which were easy to occur in the absence of urea. From above results, I confirmed that the formation yield of native like molecule by mixing the peptide fragment of 59 ~ 105 and the residual peptide fragment of lysozyme had an optimum concentration with two limbs, association phase and aggregation phase between these peptide fragments. Thus, under the condition where the formation yield of native like molecule was at the maximum, the following association experiments between the peptide fragments were carried out.

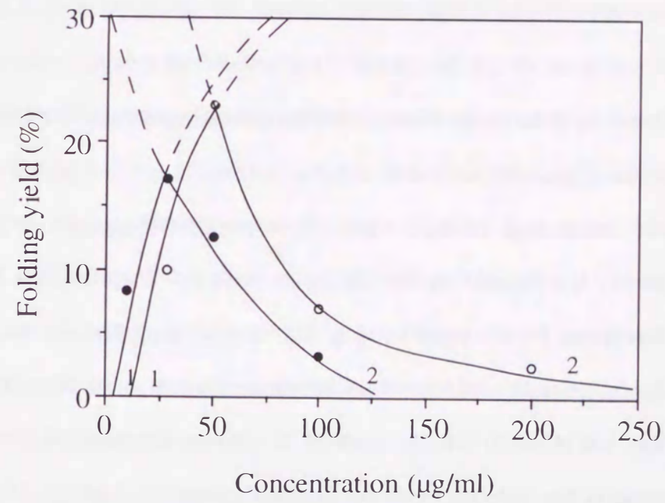

Figure III-2 The formation yield of native like structure on the association of fg. 59 ~ 105 and fg. 1 ~ 58 + 106 ~ 129 in the various concentration of peptides under the condition of 1.0 M urea (open circles) or 0 M urea (closed circles). Estimated association (1) and aggregation (2) curve.

Association of the mutant peptide fragment 59 ~ 105 with the residual peptide fragment (1 ~ 58 + 106 ~ 129)

In order to deny a possibility that the formation of native like molecule by the association between the peptide fragment 59 ~ 105 and the residual peptide fragment of lysozyme in wild-type lysozyme depends on the structure of the latter peptide fragment, I prepared the peptide fragment 59 ~ 105 from the mutant where Cys 64, Cys 76, Cys 80 and Cys 94 are simultaneously mutated to Ala (4CA fragment 59 ~ 105) (See Materials and Methods) and examined whether the 4CA fragment 59 ~ 105 associated with the residual peptide fragment to form native like molecule. After association procedure of these peptide fragments, the dialysate was applied to the ion-exchange HPLC (Figure III-1B). No peak was detected on the chromatogram. The net charge in native like molecule derived from the mixture between the 4CA fragment 59 ~ 105 and the residual peptide fragment was almost identical to that of wild-type lysozyme under the HPLC condition. Therefore, it was evidenced that the mixing between the equimolar mixture of the 4CA fragment 59 ~ 105 and the residual peptide fragment did not give native like molecule. Thus, the formation of native like molecule by association between the fragment 59 ~ 105 and the residual peptide fragment was not induced by the residual peptide fragment. Moreover, the formation of native like molecule was suggested to require the presence of cystines (Cys 64 - Cys 80 and Cys 76 - Cys 94) in the peptide fragment 59 ~ 105.

On the other hand, Trp 62 was shown to be essential to correct formation of two cystines (Cys 64 - Cys 80 and Cys 76 - Cys 94) in the reoxidation of reduced peptide fragment 59 ~ 105 (CHAPTER II). In order to evaluate the effect of Trp 62 on the formation of native like molecule in the association experiments, I prepared the fragment 59 ~ 105 from the mutant where Trp 62 is mutated to Ala (W 62 G fragment 59 ~ 105) (See Materials and Methods) and examined whether the W 62 G fragment 59 ~ 105 associated with the residual peptide fragment of wild-type lysozyme to form native like molecule. After association of these peptide fragments, the dialysate was applied to the

ion-exchange HPLC (Figure III-1C). No peak was detected on the chromatogram. The net charge in native like molecular derived from the mixture between the W 62 G fragment 59 ~ 105 and the residual peptide fragment of wild-type lysozyme was almost identical to that of wild-type lysozyme. Moreover, the dialysate did not show lytic activity whereas W 62 G lysozyme had small lytic activity. As a result, I concluded that the association between the equimolar mixture of the W 62 G fragment 59 ~ 105 and the residual peptide fragment did not give native like molecule.

The fluorescence spectra of the peptide fragment 59 ~ 105, the 4CA fragment 59 ~ 105 and the W 62 G fragment 59 ~ 105 in the presence of 1.0 M or 8.0 M urea are shown in Figure III-3. Tryptophyl fluorescence represents the environment of tryptophan residue. When the tryptophan residue in lysozyme is exposed to the solvent, the tryptophyl fluorescence shows a maximum wavelength at 354 nm. However, the maximum wavelength of the tryptophyl fluorescence generally shifts to blue as it is shielded from the solvent. As the tryptophyl fluorescence of the peptide fragment 59 ~ 105 showed a maximum wavelength at 348 nm in the presence of 1.0 M urea and a maximum wavelength at 354 nm in the presence of 8.0 M urea, the peptide fragment 59 ~ 105 was considered to have some tertiary structure in the solution containing 1.0 M urea. On the other hand, the tryptophyl fluorescence of the 4CA peptide fragment 59 ~ 105 and the W 62 G peptide fragment 59 ~ 105 showed the maximum wavelength at 354 nm even in the presence of 1.0 M urea, showing that these peptide fragments did not have some tertiary structure in 1.0 M urea. From these results, the formation of native like molecule by the association of the equimolar mixture of the fragment 59 ~ 105 and the residual peptide fragment of lysozyme required the presence of two cystines (Cys 64 - Cys 80 and Cys 76 - Cys 94) in the peptide fragment 59 ~ 105 and such conformation of the peptide fragment 59 ~ 105 that tryptophan residues were shielded from the solvent. Therefore, this result strongly suggested that the peptide fragment 59 ~ 105 folded in the early stage

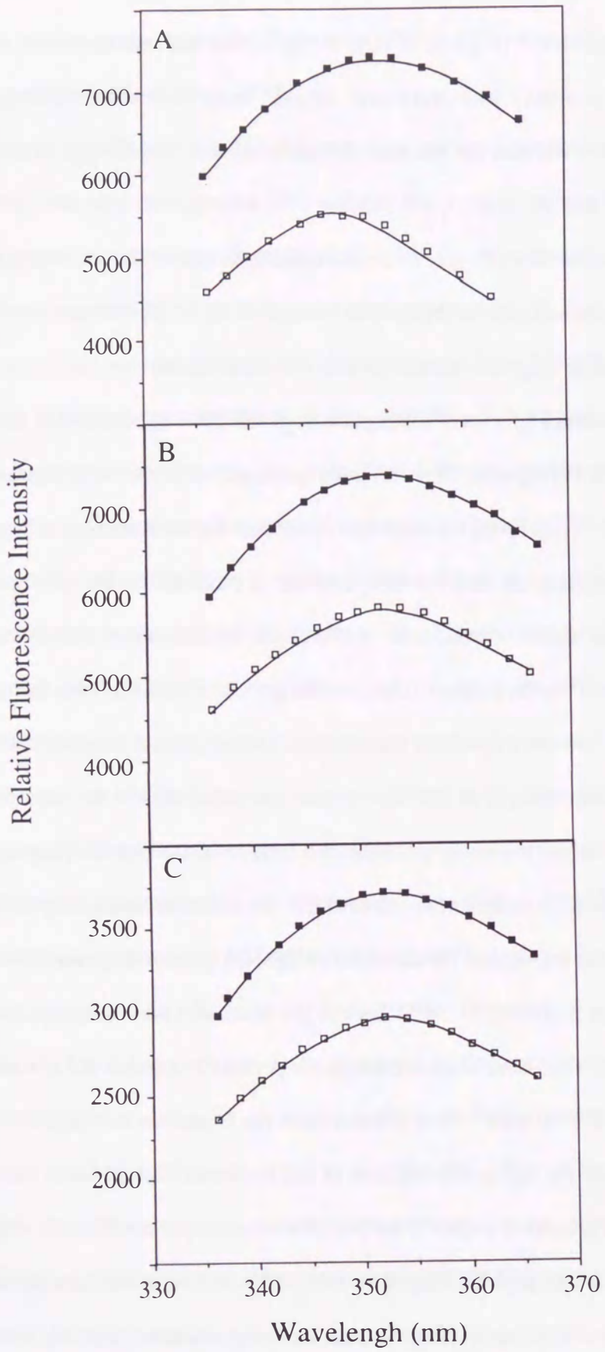

Figure III-3 The fluorescence spectra of fg. 59 ~ 105 (A), 4CA fg. 59 ~ 105 (B), and W 62 G fg. 59 ~ 105 (C) in the presence of 1.0 M urea (open squares) or 8.0 M urea (close squares) on excitation at 280 nm in 0.1 M Tris-HCl buffer (pH 8.0).

of the folding of lysozyme from the reduced form, and that the formation of fg. 59 ~ 105 was involved in the formation of structure required for the manifestation in folding process of lysozyme.

Renaturation of the mutant lysozyme where Cys 6, Cys 30, Cys 115 and Cys 127 are simultaneously mutated to Ala from its reduced form

In order to elucidate whether the result obtained by using peptide fragments can be applied to whole molecule of lysozyme, I prepared the mutant lysozyme where Cys 6, Cys 30, Cys 115 and Cys 127 are simultaneously mutated to Ala (C 6 A C 30 A C 115 A C 127 A) by using *Escherichia coli* expression system and examined the renaturation from its reduced form (See Materials and Methods). As N-terminal residue of hen lysozyme is lysine, Met residue remains at N-terminal of hen lysozyme expressed in *Escherichia coli*. Since the extra Met residue remained at N-terminal on hen lysozyme was reported to cause the lower folding yield and solubility of lysozyme, I designed the mutant lysozyme so that Ser residue remains at N-terminal of hen lysozyme (Ser^{-1} lysozyme) according to the literature (Mine *et al*, 1997) (See Materials and Methods). The unfolded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme purified from gel chromatography (500 μg) was dissolved in 5 ml of 8.0 M urea solution. To the solution, 25 μl of 2-mercaptoethanol was added and incubated at 40°C for 60 min for reduction. To the reduced solution, 81 mg of GSSG was added and incubated at 40°C for 20 min. The solution was dialyzed against the renaturation buffer by using the renaturation device according to the previous reports (Maeda *et al*, 1995) (See Materials and Methods). After folding of the reduced Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme, the pH of the supernatant of dialysate was lowered to 5.5 by the addition of acetic acid and *N*-ethylmaleimide was added to the dialyze so that the concentration of *N*-ethylmaleimide became 10 mM. Purification of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme was carried out using reversed phase column, and then the collected

protein fraction was lyophilized (See Materials and Methods). The formation of disulfide bond of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme was examined according to the method of CHAPTER I. Figure III-4 shows the tryptic peptide map derived from the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme on RP-HPLC. The retention time of peptide in peak-b and the ratio of the amount of the peptide in peak-a (peptide Phe 34 ~ Arg 45) to that in peak-b were similar to those of the authentic peptide Trp 62 ~ Arg 68 plus Asn 73 ~ Lys 96 where Cys 64 - Cys 80 and Cys 76 - Cys 94 are correctly formed. Moreover, after the peptide in peak-b was digested with both α–chymotrypsin and prolylendopeptidase, the resulting product was analyzed on RP-HPLC. As a result, Cys 64 - Cys 80 and Cys 76 - Cys 94 were confirmed to be correctly formed (data not shown). Therefore, it was found that the Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme folded from the reduced form had correct pair of disulfides,

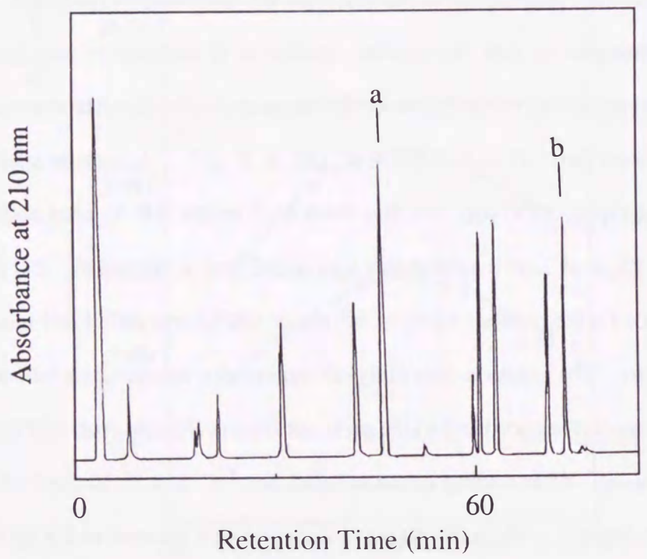

Figure III-4 The elution pattern of the tryptic peptide derived from the refolded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme on RP-HPLC. Peak-a is peptide Phe 34 ~ Arg 45, and Peak-b is peptide Trp 62 ~ Arg 68 + Asn73 ~ Lys96. The column was eluted with a gradient of 40 ml of 1% acetonitrile and 40 ml of 40% acetonitrile both containing 0.1% HCl.

Cys 64 - Cys 80 and Cys 76 - Cys 94. The folding yield of the reduced Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme was determined to be 30% at a concentration of 100 μg/ml of Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme. Thus, I examined the effect of the concentration of Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme on the folding yield (Figure III-5). The folding yield was determined by the amount of the correct disulfide bond in the supernatant of Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme formed in the dialysate. The folding yield of Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme at a concentration of 200 μg/ml was less than 10% whereas that at a concentration of 25 μg/ml was about 40%. These results indicated that the folding yield of the reduced Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme depended on the lysozyme concentration whereas that of the reduced Ser^{-1} lysozyme did not under the condition employed.

Figure III-6 shows the dependency of the tryptophyl fluorescence intensity of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme on Gdn-HCl concentration. The transition was broad but there was clear difference in the change of tryptophyl fluorescence between the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme and N-acetyl tryptophan ethylester, a model compound. The result indicated that the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme had some tertiary structure in solution without denaturants. The apparent transition concentration was around 2.0 M Gdn HCl. Since the midpoint of the folded Ser^{-1} lysozyme against Gdn-HCl denaturation was reported to be 2.8 M (Mine *et al*, 1997), the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme was found to be less stable than the folded Ser^{-1} lysozyme.

Addition of 20% glycerol was reported to increase the folding yield of unfolded proteins (Sawano *et al*, 1992). Therefore, the folding of the reduced Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme was carried out at a concentration of 25 μg/ml in the presence of 20 % glycerol. The folding yield of the reduced Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme in the presence of 20% glycerol was 62%, which is much higher than that in

the absence of glycerol. Therefore, the reason why the folding yield of the reduced Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme was low was suggested to be due to its intrinsic low stability, resulting in high occurrence of the aggregation. However, the folding yield of Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme in the presence of 20% glycerol (62%) was lower than that of Ser^{-1} lysozyme. Ser^{-1} lysozyme employed here was once folded and purified on ion-exchange chromatography in order to remove impurity. In my laboratory, it was shown that there were many impurities like deamidated species in lysozyme from *E. coli* extract (Maeda *et al*, 1998). Considering above results, I could judge that the Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme could form some tertiary structure from the reduced form.

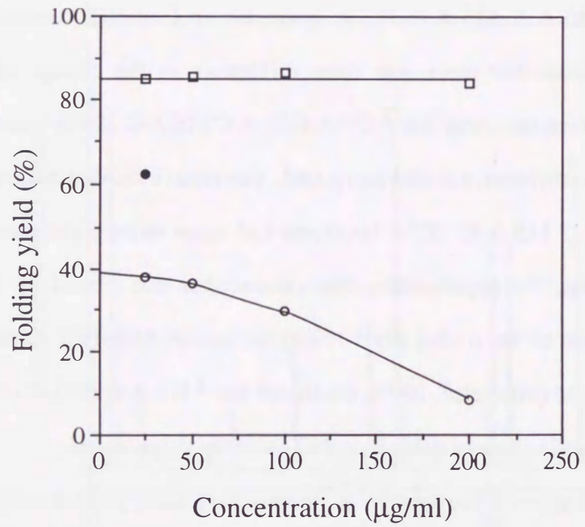

Figure III-5 The folding yield of the reduced Ser^{-1} C 6 A C 30 A C 1 15 A C 127 A lysozyme in the absence of 20% glycerol (open circles), in the presence of 20% glycerol (close circle) and Ser^{-1} lysozyme which was once refolded and purified on ion-exchange chromatography (open square).

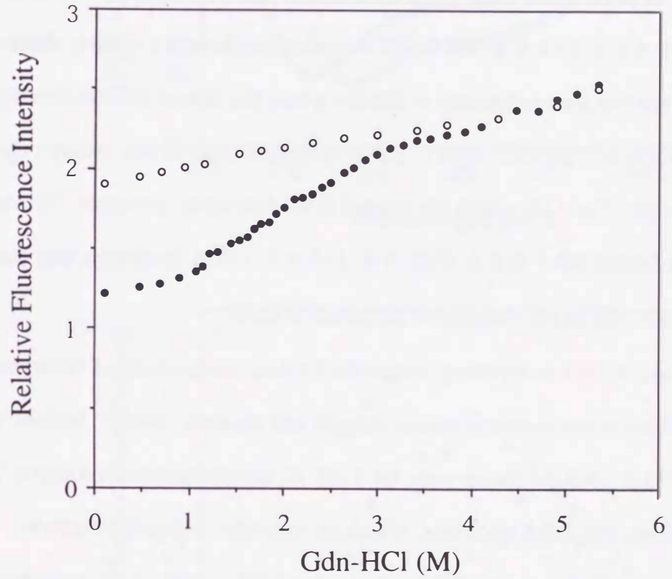

Figure III-6 The fluorescence intensity (360 nm) of the refolded C 6 A C 30 A C 115 A C 127 A Ser^{-1} lysozyme (closed circles) and N-acetyl tryptophan (open circles) with an increase of Gdn-HCl concentration on excitation at 280 nm in 0.1 M sodium acetate buffer (pH 5.5) at 35°C.

Solution behavior of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme

From the tryptophyl fluorescence spectrum on Gdn HCl denaturation, it was suggested that the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme has some tertiary structure in aqueous solution. In order to characterize the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme in solution further, CD spectra of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme and the wild-type lysozyme in the absence or presence of 6.0 M Gdn HCl at pH 8.0 and 20°C were measured (Figure III-7A). The spectrum of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme showed a similar shape with native lysozyme but had lower ellipticity at 222 nm where the helical content is reflected. The folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme lacks in two cystines Cys 6 - Cys 127 and Cys 30 - Cys 115, which are located in α–domain of lysozyme. Therefore, α–domain in the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme may possess loose structure, resulting in the decrease of the helical content.

On the other hand, ANS was reported to bind the folding intermediate of lysozyme but not lysozyme folded to the native structure (Joseph and Nagaraj, 1992). Recently, we also have found that ANS binds the reduced *S*-*N*, *N*, dimethylaminoiopropyl-*N* ', *N* ', *N* '-trimethyl aminopropylated lysozyme, which is a soluble reduced lysozyme, from analysis of fluorescence spectrum (Ueda *et al.* unpublished result). I measured the fluorescence spectrum of the reduced *S*-*N*, *N*, *N*-trimethylaminopropylated lysozyme (TAP lysozyme), which is also a soluble reduced lysozyme (Okazaki *et al.*, 1985), or the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme in the presence of ANS. The fluorescence spectrum of ANS in the presence of TAP lysozyme showed a typical pattern where the maximum wavelength is around 480 nm as seen in the binding of ANS to the folding intermediate of a protein (Figure III-7B). The fluorescence spectrum of the ANS in the presence of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme did not show pattern similar to that of TAP lysozyme (Figure III-7B). However, as its

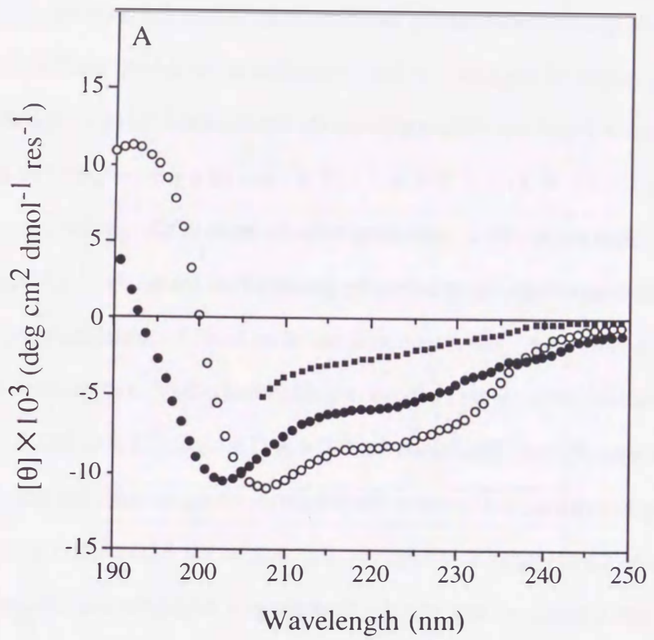

Figure III-7 A, The CD spectra of the refolded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme (closed circles) and wild-type lysozyme in the absence (open circles) or presence (closed squares) of 6 M Gdn-HCl at pH 8.0 and 20°C.
B, The fluorescence spectrum of the reduced TAP lysozyme (open squares), wild-type lysozyme (open circles), and the refolded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme (closed circles) in the presence of ANS 100 mM on excitation at 365 nm.

fluorescence spectrum was clearly different from that in the presence of the wild-type lysozyme, ANS was suggested to bind somewhat to the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme. This suggested that hydrophobic surfaces in some parts of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme were exposed to the solvent so that ANS might weakly bind, consisting with the result of CD spectra.

Figure III-8 shows gel chromatography pattern of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme. The retention times of reduced S-carboxymethylated lysozyme where disulfide bonds were reduced and alkylated (CM-lysozyme) and the wild-type lysozyme were shown. The folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme had a larger hydrodynamic volume than the wild-type lysozyme but a smaller hydrodynamic volume than CM-lysozyme. Moreover, the peak on the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme was broader than those of wild-type and CM lysozyme. This indicates that molecule with different conformations in the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme may be present in solution. This broadness may be involved in its broad transition on Gdn-HCl denaturation. The result obtained by gel chromatography was consistent with the above results that the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme had a loose helical structure than the wild-type lysozyme and that ANS somewhat bound to the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme.

A three-disulfide derivative of hen lysozyme was made by selective reduction and carboxymethylation of one of the four original disulfide bridges (Cys 6,127 CM-lysozyme) and it retained the same secondary and tertiary structure as the wild-type lysozyme. And the transition was still highly co-operative (Radford. *et al*, 1991). As was shown above, the structure of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme was different from that of the wild-type lysozyme and its transition was less co-operative. Therefore, a disulfide bond Cys 30 - Cys 115 would cause the drastic difference in the maintenance of tertiary structure from Cys 6,127 CM-lysozyme to the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme.

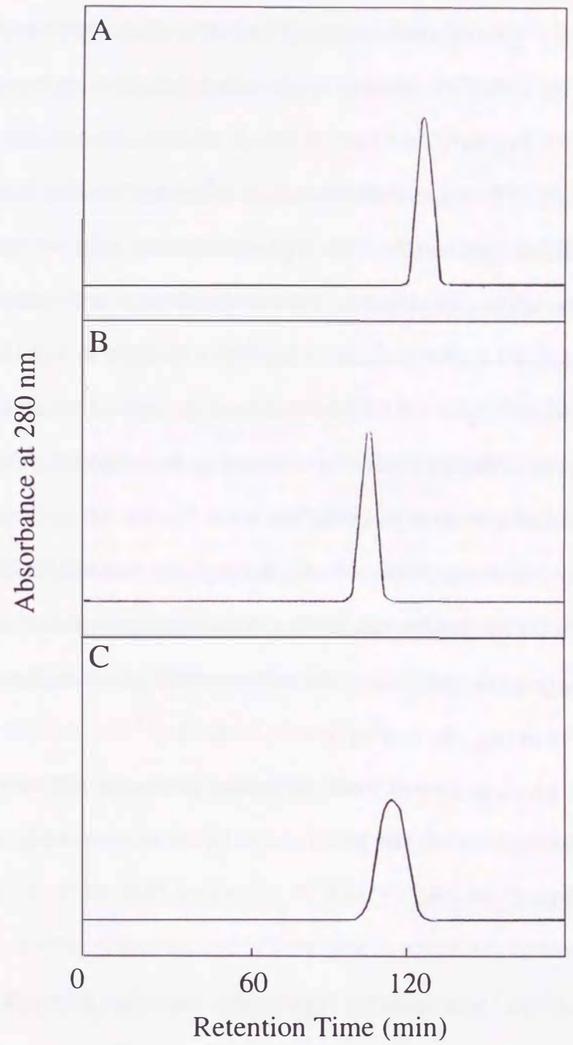

Figure III-8 Gel chromatography pattern of wild-type lysozyme(A), reduced CM lysozyme (B), and the refolded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme (C). The column was eluted with 10% aqueous acetic acid at a flow rate of 0.3 ml/min.

Activity of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme

As described above, I showed that the reduced lysozyme without cystines Cys 6 - Cys 127 and Cys 30 - Cys 115 could form correct cystines Cys 64 - Cys 80 and Cys 76 - Cys 94 and have some tertiary structure. In order to elucidate the contribution of the formation of peptide region between position 59 and 105, which formed in the early stage of the folding of the reduced lysozyme, to the manifestation of lysozyme activity, I measured lytic activity of the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme against *M. luteus* at 30°C. A hundred microliter of folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme dissolved in 0.05 M Na-K phosphate buffer (pH 7.0) was added to 2 ml of the suspended solution of *M. luteus*, and then the decrease in the absorbance at 450 nm was measured. As a result, the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme had a lytic activity of 1.0 % of the wild-type lysozyme. Acharya and Taniuchi have demonstrated that only the reduced and S-carboxymethylated Cys 30 and Cys 115 lysozyme had no lytic activity in one disulfide bond cleaved lysozymes that had been obtained by trapping the folding intermediates (Acharya and Taniuchi, 1972). This result was inconsistent with the present result. The discrepancy between these results may depend on the charge or the bulkiness of the carboxymethyl group or that they neglected this low activity. Anyway, the finding that the folded Ser^{-1} C 6 A C 30 A C 115 A C 127 A lysozyme, which lacks two disulfide bonds Cys 6 - Cys 127 and Cys 30 - Cys 115, does have the activity will be novel and help the understanding of the folding process of the reduced lysozyme.

Significance of the hierarchical approach on the analysis of folding process of proteins

First, I presented in this CHAPTER that the association between the peptide fragment 59 ~ 105 and the residual peptide fragment of lysozyme resulted in the formation of native like molecule. Based on the information, I designed a mutant lysozyme where Cys

6, Cys 30, Cys 115 and Cys 127 are simultaneously mutated to Ala, and renatured it from the reduced form. By this hierarchical approach, I could obtain useful information on the folding process of the reduced lysozyme. As for some proteins with disulfide bonds such as BPTI (Creighton 1990; Weissman and Kim 1991), ribonuclease T1 (Pace and Creighton, 1986; Schonbrunner and Schmid, 1992) and so on, intermediates in the folding process of the reduced proteins could have been separated because these proteins have at most three disulfide bonds. By analyzing the characters of intermediates such as disulfide bonds and activities, the folding process of the reduced proteins could be discussed. However, as lysozyme has four cystines, it would be hard to separate the folding intermediates from the reduced lysozyme due to the presence of many intermediates. As for proteins with four disulfide bonds, a challenge to analyze the intermediates in the folding of the reduced ribonuclease A has been carried out by Scheraga's group (Rothwarf and Scheraga, 1991) but the resulting information of the folding process from the reduced one was limited in comparison with those of the simple proteins such as BPTI and ribonuclease T1. In this meaning, the present hierarchical approach may be laborious but steady to obtain the information of the folding process from the reduced proteins with several disulfide bonds.

Comparison of the present result with the others

Under non-reduced condition, Dobson's group has demonstrated that α–domain of lysozyme forms earlier than β–domain of lysozyme by using pulse labeling NMR (Radford *et al*, 1992). Alpha-domain of lysozyme contains two cystines Cys 6 - Cys 127 and Cys 30 - Cys 115, β–domain contains a cystine Cys 64 - Cys 80 and a cystine Cys 76 - Cys 94 connects the interdomain. In the folding of the reduced lysozyme, I suggested that Trp 62 and/or Trp 63 interacted with the C-helix (88 ~ 98) (Ueda *et al*, 1994) and the peptide region 59 ~ 105 formed earlier than the other region of lysozyme in the folding of the reduced lysozyme (CHAPTER II). These indicated that disulfide

bonds in α–domain of lysozyme did not always form earlier than that in β–domain in the folding of reduced lysozyme. Moreover, the present result confirmed the involvement of the peptide region 59 ~ 105, where two cystines Cys 64 - Cys 80 and Cys 76 - Cys 94 are located, in the manifestation of lysozyme activity.

Although, Chaffote *et al* (1992) reported that helical structures of lysozyme formed within a few millisecond under non-reducing condition. Moreover, formation rate of helical structure was reported to be different between the presence and the absence of disulfide bond (Goldberg and Guillou, 1994). Therefore, the folding process of lysozyme between reducing condition and non-reducing condition may be different. In this meaning, hereafter, we should consider to discriminate the folding process of lysozyme between under non-reduced condition and under reduced condition.

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CHAPTER IV

Kinetic Analysis of Folding of Reduced Lysozyme Possessing Ca^{2+} Binding Site

ABSTRACT

In chapter II and III, I found that the formation of region between 59 and 105 was cue in the folding process of the reduced hen lysozyme. A Ca^{2+} binding loop region in human α-lactalbumin was designed in hen lysozyme by substituted the corresponding sequence, 82 ~ 91, to that of α-lactalbumin by site-directed mutagenesis (CaB lysozyme). The folding of the CaB lysozyme from reduced form was analyzed in order to examine the effect of Ca^{2+} on the folding rate. The folding rate of reduced Ser^{-1} CaB lysozyme in the presence of 2 mM EDTA was slower than that of wild-type lysozyme, but that in the presence of 2 mM $CaCl_2$ was faster than that of wild-type lysozyme. These results indicated that the binding of Ca^{2+} accelerated the folding process of reduced lysozyme. Therefore, it was concluded that the formation of the loop 82 ~ 91 included in the region 59 ~ 105 closely participated in the early stage of lysozyme folding.

INTRODUCTION

Hen lysozyme and α-lactalbumin are evolutionary related proteins, as evidenced by the similarity of amino acid sequences (Brew *et al*, 1970) and the tertiary structures (Smith *et al*, 1987). Alpha-lactalbumin has a Ca^{2+} binding site, but hen lysozyme dose not. The Ca^{2+} binding site is composed of three critical aspartate (Asp 86, Asp 91, and Asp 92) and two main-chain carbonyl groups (Stuart *et al*, 1986). The tertiary structure of α–lactalbumin is stabilized by Ca^{2+} (Hiraoka et al, 1980; Kronman et al, 1981). Moreover,

the folding rate of α–lactalbumin was accelerated by Ca^{2+} under non-reduced condition (Kuwajima *et al*, 1989). The Ca^{2+} binding site of α–lactalbumin is located in the region corresponding to the 82 ~ 91 loop region in hen lysozyme. The 82 ~ 91 loop region is located within 59 ~ 105 region, which forms in the early stage of lysozyme folding. Figure IV-1 shows the amino acid sequence of the Ca^{2+} binding site of human α-lactalbumin and the corresponding sequence of hen lysozyme. Thus, I prepared the mutant lysozyme where the amino acid residues between position 82 and position 91 in hen lysozyme are mutated to Lys-Phe-Leu-Asp-Asp-Asp-Ile-Thr-Asp-Asp (CaB lysozyme), the Ca^{2+} binding site of human α-lactalbumin (Figure IV-1). In this CHAPTER, by using CaB lysozyme, I examined the effect of Ca^{2+} on the folding rate of lysozyme.

	82									91
Hen Lysozyme	Ala	Leu	Leu	Ser	Ser	Asp	Ile	Thr	Ala	Ser
	79									88
Human α–Lactalubumin	Lys	Phe	Leu	Asp	Asp	Asp	Ile	Thr	Asp	Asp
	79									88
CaB Lysozyme	Lys	Phe	Leu	Asp	Asp	Asp	Ile	Thr	Asp	Asp
	*			**		*			**	**

Figure IV-1 Sequence alignments of loop region in hen lysozyme, CaB lysozyme, human α-lactalbumin, and bovine α-lactalbumin were cited from Kuroki *et al.* (1989). Position of backbone carbonyl ligand (*). Position of the side-chain carboxyl ligand (**).

MATERIALS AND METHODS

Materials

Hen egg-white lysozyme, *Micrococcus luteus*, a column of Cosmosil C18 were obtained as shown in the previous CHAPTER. All other chemicals used were of the highest quality commercially available.

Site-directed mutagenesis of hen lysozyme

Site-directed mutagenesis was performed as shown in CHAPTER II. The mutations were confirmed by DNA sequencing.

Expression of Ser^{-1} CaB lysozyme

Expression and purification of Ser^{-1} CaB lysozyme were performed with an expression vector pET22b (+) in *E. coli* BL21 (DE3) cells as shown in CHAPTER III. The inactive Ser-^{1} CaB lysozyme derived from the inclusion body were renaturated to active forms under condition in the presence of 100 mM $CaCl_2$ and absence of EDTA according to the methods of CHAPTER III. The renatured Ser-^{1} CaB lysozyme was purified as following. The pH of the solution was lowered to 5.5 with acetic acid and then 10 mM *N*-ethylmaleimide was added to the solution. After stirring for 30 min, the pH of the solution was adjusted to 3.0. The solution was applied directly to reversed-phase column (Cosmosil C18, 1.5 mm x 150 mm) and eluted with a liner gradient from 50 ml of 10% acetonitrile to 50 ml of 60% acetonitrile, both containing 0.1% acetic acid. The eluted fractions were collected and applied to a CM-Toyopearl cation-exchange column which was eluted with a gradient of 200 ml of 0.1 M sodium acetate buffer (pH 5.0) and 200 ml of the same buffer containing 0.5 M NaCl. The eluted Ser^{-1} CaB lysozyme was dialyzed against distilled water and lyophilized.

The folding of reduced lysozymes by means of the rapid dilution

The folding of reduced lysozymes were carried out according to the previous paper (Ueda *et al*, 1990) with a slight modification. One milligram of wild-type lysozyme or Ser^{-1} CaB lysozyme was dissolved in 8 M urea solution (pH 8.0) with 2 mM $CaCl_2$ or 2 mM EDTA. 2-Mercaptoethanol was added to the solution and the solution was incubated at 40°C for 1 h (reduced solution). On the other hand, 19.8 ml of renaturation buffer ((0.1M Tris-HCl containing 2 mM $CaCl_2$ or 2 mM EDTA (pH 8.0))) containing 8.1 mg GSSG was preincubated at 40°C. Folding of the reduced lysozyme was initiated by adding 200 μl of the reduction solution to the renaturation buffer with stirring, and was monitored by following the recovery of the lytic activity according to the literature (Anderson *et al.*, 1976). At appropriate time intervals up to 60 min, aliquots of the folding mixture were transferred into 2 ml of suspension of *Micrococcus luteus* (0.25 mg/ml) in 0.05 M Na-K phosphate buffer (pH 7.0) and initial rates of decrease in turbidity were measured at 450 nm.

RESULTS AND DISCUSSION

Expression and Purification of Ser^{-1} CaB lysozyme in *E. coli*

I produced CaB lysozyme by using *E. coli* expression system because a large amount of lysozyme could be obtained using this system. Expression and purification of Ser^{-1} CaB lysozyme from the inclusion bodies produced by *E. coli* were carried out as shown in CHAPTER III. The renaturation of the inactive Ser^{-1} CaB lysozyme derived from the inclusion body were renaturated to active form as shown in CHAPTER III. The renatured Ser^{-1} CaB lysozyme derivatives were purified by means of reversed-phase column and then ion-exchange. The pattern of ion-exchange is shown in Figure IV-2. The eluted Ser^{-1} CaB lysozyme was dialyzed against distilled water and lyophilized. The yields of renatured Ser^{-1} CaB lysozyme were approximately 9 mg 1 L culture.

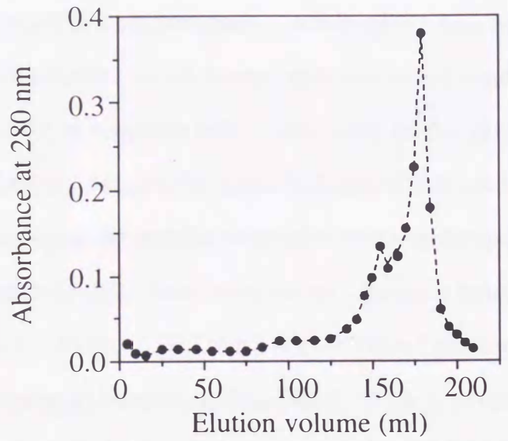

Figure IV-2 Ion-exchange chromatography of the renatured Ser^{-1} CaB lysozyme on CM-Toyopearl (1.2 x 50 cm). The column was eluted with a gradient of 200 ml of 0.1 M sodium acetate buffer (pH 5.0) and 200 ml of the same buffer containing 0.5 M NaCl.

The folding of reduced Ser^{-1} CaB lysozyme and wild-type lysozyme

Ser^{-1} CaB lysozyme and wild-type lysozyme were reduced and then folded by SH-SS interchange reaction, catalyzed by 2-mercaptoethanol and GSSG at a concentration of 20 μg/ml. Folding of the reduced Ser^{-1} CaB lysozyme was carried out in the presence of 2 mM $CaCl_2$ or 2 mM EDTA. The time courses of the recoveries of the lytic activities (percentages with respect to the original activities of the respective lysozymes) are shown in Figure IV-3. These foldings of the reduced lysozymes obeyed first-order kinetics. Under the condition employed, the deviation of the recovery of activity for native lysozyme was less than 5%.

The folding yield of reduced Ser^{-1} CaB lysozyme in the presence of 2 mM EDTA was lower than that of wild-type lysozyme. In the presence of 2 mM EDTA, Ca^{2+} could not bind to Ser^{-1} CaB lysozyme because a trace amount of Ca^{2+} could be trapped by EDTA. Maeda *et al.*(1994) suggested that the decrease in the net charge of lysozyme led to

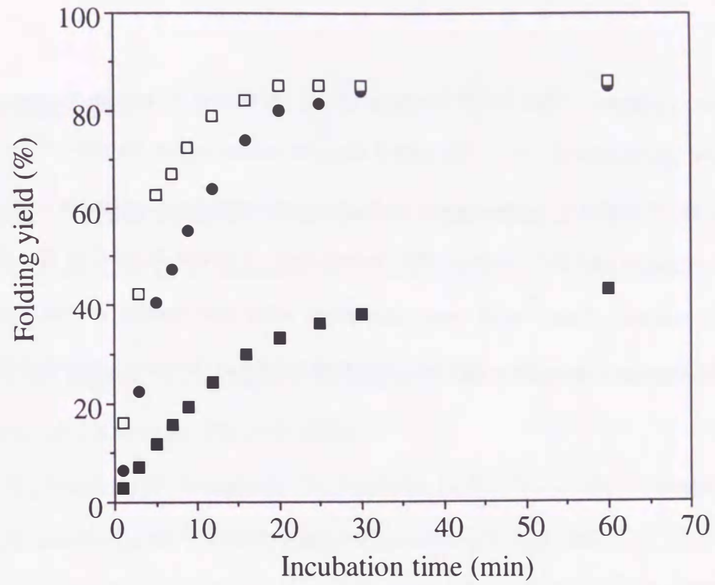

Figure IV-3 The folding of the reduced lysozyme by SH-SS interchange reaction at pH 8.0 and 40°C. Wild-type lysozyme (closed circles). Ser^{-1} CaB lysozyme in the presence of 2 mM $CaCl_2$ (opened squares) and 2 mM EDTA (closed squares).

decrease in the folding yield of the reduced lysozyme by depressing the electrostatic repulsion of the unfolded lysozyme and causing aggregation. Therefore, since the positive net charge of Ser^{-1} CaB lysozyme was smaller than that of wild-type lysozyme, the folding yield of Ser^{-1} CaB lysozyme in the presence of 2 mM EDTA would decrease. The folding rate of reduced Ser^{-1} CaB lysozyme in the presence of 2 mM EDTA was slower than that of wild-type lysozyme. The Ca^{2+} binding site of Ser^{-1} CaB lysozyme are located within the region which formed on the early stage of reduced lysozyme. The reason why the decrease in folding rate of reduced Ser^{-1} CaB lysozyme in the presence of 2 mM EDTA would depend that the electrostatic repulsion at the introduced Ca^{2+} binding site depressed the folding of reduced lysozyme.

On the other hand, the folding rate of reduced Ser^{-1} CaB lysozyme in the presence of 2 mM $CaCl_2$ was faster than that of wild-type lysozyme. This result indicated that the binding of Ca^{2+} accelerated the folding process of lysozyme. In CHAPTER III, I found that the formation of region between 59 and 105 was cue in the folding process of the

reduced hen lysozyme. The Ca^{2+} binding site is located at loop region connecting between α–domain (α-helix 88 ~ 98) and β-domain in the region 59-105 of lysozyme (Figure IV-4). Therefore, the formation of loop region would accelerate the interaction between α–domain and β-domain so that interdomain disulfide bond Cys 76 - Cys 94 might form easily. This result was consistent with the results in the previous CHAPTERS that the region 59 ~ 105 was deeply involved in the early stage of lysozyme folding.

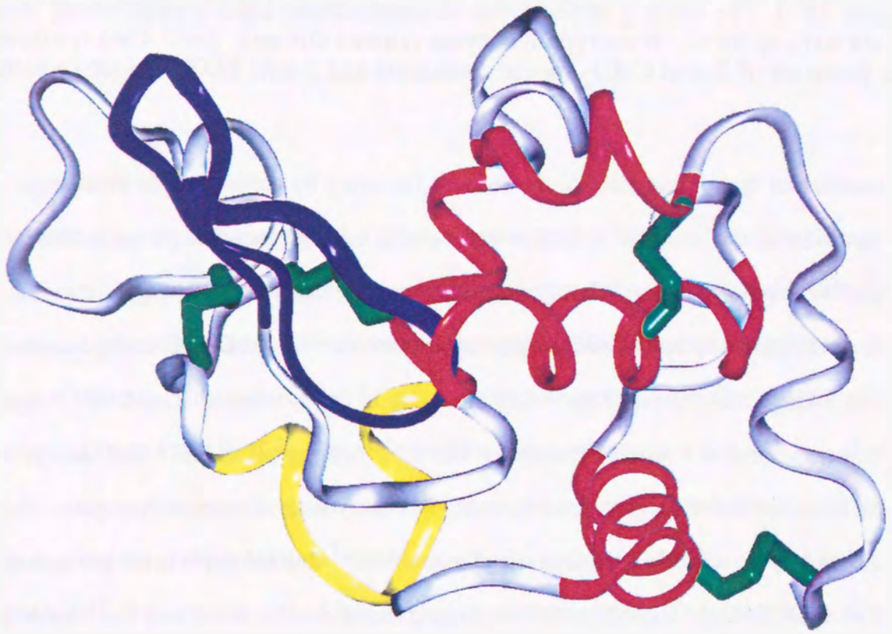

Figure IV-4 The tertiary structure of hen lysozyme. The yellow line indicates the loop corresponding to the Ca^{2+} binding site of CaB lysozyme. The blue line indicates the β-sheet. The red lines indicate the α-helix. The green lines indicate the disulfide bonds.

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CHAPTER V

Efficient Folding of Reduced Hen Lysozyme Possessing a Ca^{2+} Binding Site in the Presence of Highly Concentrated Ca^{2+}

ABSTRACT

In CHAPTER IV, I prepared hen lysozyme possessing a Ca^{2+} binding site by site-directed mutagenesis (CaB lysozyme). The folding yield of the CaB lysozyme from reduced form was analyzed in order to examine the effect of Ca^{2+} on the aggregation. In the presence of 2 mM $CaCl_2$, the folding yield of Ser^{-1} CaB lysozyme at a low protein concentration (25 μg/ml) was similar to that of wild-type lysozyme (80%), but that at a high concentration (200 μg/ml) decreased (15%) due to aggregation comparing to that of wild-type lysozyme (45%). However, the folding yield in the presence of 100 mM $CaCl_2$ even at a concentration of 200 μg/ml was 80% and was higher than that of the wild-type lysozyme. Therefore, it was concluded that the reduced lysozyme possessing a Ca^{2+} binding site was efficiently folded in the presence of high concentration of Ca^{2+} even at high lysozyme concentration. This idea would be applicable to the efficient renaturation of the reduced other proteins carrying Ca^{2+} binding sites.

INTRODUCTION

By use of *Escherichia coli* expression system, most of the desired proteins could be produced. This expression system has an advantage over the other expression system in growth speed, production yield of proteins and so on. However, in this system, proteins are often produced as inclusion bodies in their inactive forms, and we must renature them *in vitro*. The yield of folded protein is usually low at a higher concentration of protein

due to occurrence of aggregation. Goldberg *et al.* (1991) have demonstrated that the folding of the reduced lysozyme involved the kinetic competition of folding and aggregation.

In CHAPTER IV, I have shown that the folding rate of Ser^{-1} CaB lysozyme, where the Ca^{2+} binding site located on the loop region 82 ~ 91 included in region 59 ~ 105 folded in early folding stage of reduced lysozyme was faster in the presence of 2 mM $CaCl_2$ than that of wild-type lysozyme. Therefore, I wonder if acceleration of the formation of the region would depress the aggregation, resulting in the efficient folding of reduced lysozymes. However, there was no trial from the view of the efficient folding of reduced lysozyme. In CHAPTER V, by using Ser^{-1} CaB lysozyme, I examined the folding yield from its reduced form in the absence or presence of Ca^{2+}. As a result, I found that the folding yield of reduced lysozyme possessing the Ca^{2+} binding site was higher in the presence of Ca^{2+} than in the absence of Ca^{2+}, moreover, that it was higher than that of wild-type lysozyme.

MATERIALS AND METHODS

Materials

Hen egg-white lysozyme, *Micrococcus luteus* , and Cosmosil C18 were obtained as shown in the previous CHAPTER. All other chemicals used were of the highest quality commercially available.

Site-directed mutagenesis of hen lysozyme

Site-directed mutagenesis was performed as shown in CHAPTER II. The mutations were confirmed by DNA sequencing.

Expression and purification of Ser^{-1} lysozyme and Ser^{-1} CaB lysozyme

Expression and purification of Ser^{-1} lysozyme and Ser^{-1} CaB lysozyme was performed with an expression vector pET22b (+) in *E.coli* BL21 (DE3) cells according to the methods of CHAPTER IV. The renaturation of the inactive Ser^{-1} lysozyme and Ser^{-1} CaB lysozyme derived from the inclusion body were renaturated to active forms according to the methods of in CHAPTER IV.

Folding of reduced lysozymes by means of rapid dilution

The folding of reduced lysozymes were carried out as shown in CHAPTER IV.

RESULTS AND DISCUSSION

Effect of concentration of lysozymes on their folding yields from their reduced forms

Various concentrations of Ser^{-1} lysozyme or Ser^{-1} CaB lysozyme were reduced and then folded in the absence or presence of $CaCl_2$ or EDTA according to the method of CHAPTER IV. In all cases, the folding from reduced form completed within 60 min. However, the recoveries of the activities (folding yield) at infinite time (60 min) were not the same. The plots of the folding yields of lysozymes in several conditions at infinite time against concentration of lysozymes are shown in Figure V-1. The folding yield of Ser^{-1} lysozyme was 80% at a lysozyme concentration of 25 μg/ml, which was similar to that of wild-type lysozyme based on the previous report (Maeda *et al.*, 1994). However, with an increase of lysozyme concentration, the folding yield decreased due to aggregation which has been also observed in wild-type lysozyme in the previous papers (Goldberg *et al.*, 1991; Maeda *et al.*, 1994; Maeda *et al.*, 1995). On the other hand, the folding yield of the reduced Ser^{-1} CaB lysozyme in the presence of 2 mM EDTA and 2 mM $CaCl_2$ steeply decreased with an increase of lysozyme concentration. In the

presence of 2 mM EDTA, under the condition where Ca^{2+} may be completely removed from Ser^{-1} CaB lysozyme, the folding yield of Ser^{-1} CaB lysozyme was less than 5% at a protein concentration of 200 μg/ml. In the presence of 2 mM $CaCl_2$ where the folding rate had been accelerated, the folding yield of Ser^{-1} CaB lysozyme (18%) at the same protein concentration was a little higher than that in the presence of 2 mM EDTA (6%) whereas that of Ser^{-1} lysozyme was 30%. The folding yield of Ser^{-1} CaB lysozyme at 200 μg/ml was 80% in the presence of 100 mM $CaCl_2$. The folding yield was higher than that of wild-type lysozyme (45%) which has been obtained in the same condition without $CaCl_2$ (Maeda *et al.*, 1994). Because the folding yield of wild-type lysozyme in the presence of 100 mM or 200 mM $CaCl_2$ was similar to that in the absence of Ca^{2+}, I found that the high folding yield of Ser^{-1} CaB lysozyme was due to the Ca^{2+} binding, not due to a simple effect of Ca^{2+} concentration.

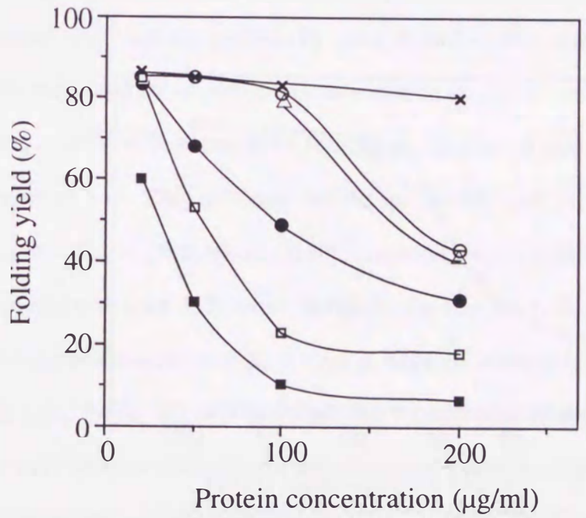

Figure V-1 The folding yield (%) of lysozymes in several conditions at infinite time against concentration of lysozymes. Ser^{-1} lysozyme (closed circles). Ser^{-1} CaB lysozyme in the presence of 100 mM $CaCl_2$ (crosses), 2 mM $CaCl_2$ (opened squares), and 2 mM EDTA (closed squares). Wild-type lysozyme (opened circles) cited from Maeda *et al.* (1994). Wild-type lysozyme in the presence of 100 mM $CaCl_2$ (triangles)

Effect of the concentration of Ca^{2+} on the folding yield of Ser^{-1} CaB lysozyme

Since there was the difference in the folding yield of Ser^{-1} CaB lysozyme in the presence of 2 mM and 100 mM $CaCl_2$, I examined the effect of the concentration of Ca^{2+} on the folding yield of reduced Ser^{-1} CaB lysozyme (Figure V-2). The folding of Ser^{-1} CaB lysozyme at a concentration of 100 μg/ml was carried out at pH 8 and 40°C in the presence of various concentrations of Ca^{2+}. The plots of the folding yield of Ser^{-1} CaB lysozyme against the concentration of Ca^{2+} gave sigmoidal curve. The transition point of the curve was approximately 35 mM Ca^{2+} concentration.

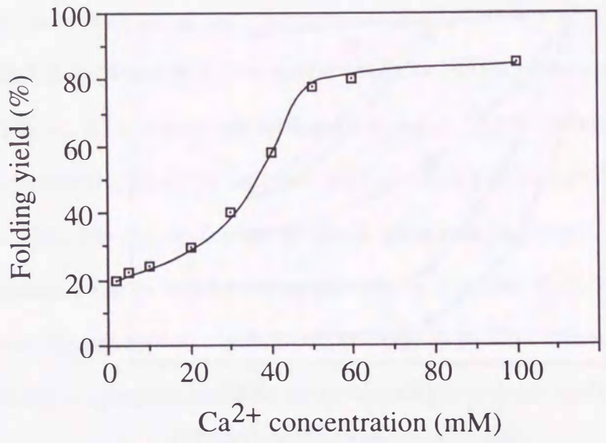

Figure V-2 The folding yield of Ser^{-1} CaB lysozyme at a concentration of 100 μg/ml in the presence of various concentrations of Ca^{2+}.

DISCUSSION

The positive net charge of Ser^{-1} CaB lysozyme was smaller than that of Ser^{-1} lysozyme. Therefore, the folding yield of Ser^{-1} CaB lysozyme in the presence of 2 mM EDTA would decrease (see CHAPTER IV). On the other hand, in the presence of 100 mM $CaCl_2$, the folding yield of the reduced Ser^{-1} CaB lysozyme even at a concentration of 200 μg/ml was higher than that of both the reduced Ser^{-1} lysozyme and wild-type lysozyme whereas the folding yield of the reduced Ser^{-1} CaB lysozyme in the presence of 2 mM $CaCl_2$ was not higher. The result indicates that the efficient folding of the reduced lysozyme closely depends on the concentration of Ca^{2+}. As there has been no report of the effect of Ca^{2+} concentration on the efficient folding of Ca^{2+} binding proteins from their reduced forms, the finding will be novel. Ca^{2+} binding proteins are widely distributed in nature and known to play significant roles. Therefore, in case of expression of Ca^{2+} binding proteins by using *E. coli* system and renaturation of them from their inactivated forms, the present information should be useful.

Goldberg *et al.* (1991) showed the folding mechanism of the reduced lysozyme as shown in Figure V-3. They indicated that the species "X" had formed in the early stage of folding of reduced lysozyme and that it caused aggregation but that a class of native like intermediates did not. Therefore, in this mechanism, the folding rate of the reduced lysozymes do not change even if the folding yield of the reduced lysozymes changes due to aggregation. Since, in the present case, the folding rates of the reduced Ser^{-1} CaB lysozyme were identical each other in the presence of 2 mM and 100 mM $CaCl_2$ (data not shown), the decrease in the folding yield of the reduced Ser^{-1} CaB lysozyme at low Ca^{2+} concentration could be explained by the above mechanism. Thus, the difference in the folding yield of reduced Ser^{-1} CaB lysozyme may involved how much Ca^{2+} binds to the intermediates which are tending to be "X" in the folding from the reduced form. Namely, it may be considered that the intermediates were harder to proceed to the species

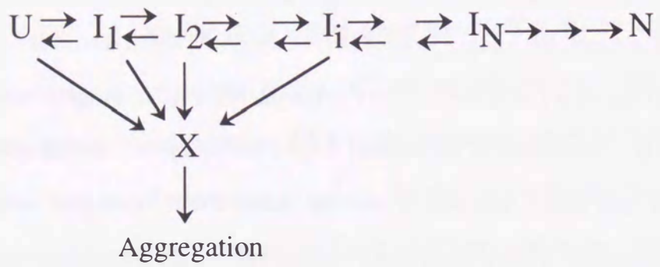

Figure V-3 Folding mechanism of the reduced lysozyme cited from Goldeberg *et al.* (1991). U, the reduced and unfolded state; I_i, intermediate; I_N, a class of native like intermediates; N, native state.

'X" in the presence of higher concentration of Ca^{2+} (100 mM) than in the presence of lower concentration of Ca^{2+} (2 mM), resulting in higher folding yield of the reduced Ser^{-1} CaB lysozyme in the presence of higher concentration of Ca^{2+}. I estimated the dissociation constant of the intermediate-Ca^{2+} complex to be approximately 35 mM.

Bovine α-lactalbumin is one of the proteins where the folding mechanism from the unfolded form under the non-reduced condition has been investigated in detail. The dissociation constant of the complex between A-state, a typical folding intermediate of bovine α-lactalbumin and Ca^{2+} was determined to be 1.1 mM (Kuwajima, 1989). The dissociation constants was reported to become smaller with the higher organization of the structure of Ca^{2+} binding site (Kuwajima, 1989). The sequence of Ca^{2+} binding site of bovine α-lactalbumin was almost identical to that of human α-lactalbumin (Figure IV-1). The difference of the amino acid residue at position 88 in Ca^{2+} binding site would not influence the binding ability of Ca^{2+} because the position was not related with Ca^{2+} binding residues (Figure IV-1). Therefore, larger dissociation constant obtained here indicated that the structure of Ca^{2+} binding site in the intermediates which are tending to

be "X" were less organized than that of A-state of bovine α-lactalbumin. From these results, it was found that Ca^{2+} weakly bound on the Ca^{2+} binding region in such a early folding stage of lysozyme from the reduced form, that intermediates which tending to be "X" in Figure IV-3 was present. Namely, it was suggested that the loop region 82 ~ 92 in lysozyme formed some tertiary structure, which was different from random structure in the initial extremely rapid folding stage of lysozyme from the reduced form.

Anyway, I showed here that the binding of Ca^{2+} to the intermediates which are tending to be "X" in the early stage of folding of the reduced Ser^{-1} CaB lysozyme depressed the aggregation, resulting in the efficient folding of the reduced Ser^{-1} CaB lysozyme.

Consequently, I found that the folding yield of reduced lysozyme possessing the Ca^{2+} binding site was higher in the presence of Ca^{2+} than in the absence of Ca^{2+}, moreover, and that it was higher than that of wild-type lysozyme. These results would be helpful to obtain a large amount of Ca^{2+} binding protein produced by *E.coli* and could significantly contribute to protein engineering.

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CHAPTER VI

Effect of Mutation at a Key Residue in the Folding of Hen Lysozyme: Evidence for the Presence of Incomplete Disulfide Bond in the Trp 62 Mutants Secreted from Yeast

ABSTRACT

I have suggested that Trp 62 is one of the key residues *in vitro* folding of hen lysozyme from reduced form (CHAPTER II and III). In order to examine whether the folding process of a protein *in vitro* is similar to that *in vivo*, the effect of the residue at the position 62 on the folding of lysozyme was analyzed by preparing a series of mutant lysozymes where Trp 62 is mutated to Phe, His, Met, Asn, and Gly, respectively. From the results of RP-HPLC, gel chromatography, and peptide analysis, it was showed that the species containing cysteine residues was present in W 62 M, W 62 N, or W 62 G lysozyme secreted from yeast, in which the residue at the position 62 was less bulky. The extent of the species in W 62 G lysozyme was estimated to be 20% of the total lysozyme. The species containing cysteine residues was trapped with amioethyl-methanthiosulfonate. Analyzing the formation of disulfide bond and cysteine, the species had two cysteines, Cys 80 and Cys 94, and a non-native cystine, Cys 64 - Cys 76. The result indicated that the folding in the early stage of lysozyme *in vivo* was resemble to that *in vitro*.

INTRODUCTION

A polypeptide chain is synthesized on ribosome according to the genetic message. The folding process is indispensable for a polypeptide chain to become a biologically active protein. However, it remains unclear whether protein folding *in vivo* is initiated as soon as a polypeptide chain is synthesized (co-translational folding) (Bergman and Kuehl, 1979; Andria and Taniuchi, 1974) or protein folding is initiated after a polypeptide chain is translated (post-translational folding) (Hartl and Neupert, 1990; Hartl *et al*, 1992; Braakman *et al*, 1992). Moreover, protein folding *in vivo* was also reported to be involved molecular chaperons such as protein disulfide isomerase and thioredoxin. On the other hand, protein folding *in vitro* is believed to be thermodynamically controlled as Anfinsen has reported (Anfinsen, 1973). At the present stage, however, we can not clearly state whether the folding process of a protein *in vitro* is similar to that *in vivo*, and we should carry out further analysis to predict this. In CHAPTER II and III, it have been suggested that Trp 62 is one of the key residues, which is closely located to Cys 64 - Cys 80, in *in vitro* folding of lysozyme from its reduced form. If the folding process of hen lysozyme *in vitro* is closely related to that *in vivo*, the mutations at the position of 62 affect the formation of disulfide bond in mutant lysozymes secreted from yeast. Therefore, I prepared a series of mutant lysozymes where Trp 62 is mutated to Gly, Asn, Met, His, or Phe, respectively, from yeast, and examined the effect of the residue 62 on the correct formation of disulfide bond *in vivo*. As a result, it was demonstrated that the species with the incompletely formed disulfide bonds existed in mutant lysozymes with a decrease in bulkiness of the residue at the position 62 secreted from yeast and that the information of protein folding obtained *in vitro* was applicable to protein folding *in vivo*.

MATERIALS AND METHODS

Materials

Hen egg-white lysozyme, a column of TSK-GEL G3000SW and Cosmosil C18, TPCK-trypsin, α-chymotrypsin, and prolylendopeptidase were obtained as shown in the

previous CHAPTER. AEMTS was the product of Sigma. All other chemicals used were of the highest quality commercially available.

Preparation, expression and purification of mutant lysozymes

Preparation, expression, and purification of mutant hen lysozymes where Trp 62 is mutated to Phe, His, Met, Asn, and Gly, respectively, and where Cys 80 is mutated to Ala were carried out as shown in CHAPTER II. The yields of these mutant lysozymes were similar to that of wild-type lysozyme (*ca.* 1 mg / L).

BrCN treatment of mutant lysozymes and their tryptic digestion

BrCN treatment of mutant lysozymes and their tryptic digestion were carried out as shown in CHAPTER I.

The determination of the extent of the correctly folded lysozyme in each mutant lysozyme at position 62

The extent of the correctly folded lysozyme was determined by the ratio to the peptide Phe 34 ~ Arg 45 in each mutant lysozyme. The amounts of these peptides were evaluated using amino acid analysis.

Gel chromatography of lysozyme

Wild-type and modified lysozymes (mutant and reduced S-carboxymethylated lysozymes) were applied to the column of TSK-GEL G3000-SW (7.5 mm x 600 mm) which was equilibrated with 10% aqueous acetic acid and eluted with the same solution at a flow rate of 0.5 ml / min.

Trapping of cysteine residue in the mutant lysozyme

W 62 G lysozyme was incubated in 0.1 M Tris-HCl buffer (pH 8.0) with AEMTS for 10 min in the presence of 8.0 M Urea.

RESULTS AND DISCUSSION

Analysis of the formation of the disulfide bonds in mutant lysozymes

In order to analyze the effect of mutations at Trp 62 on the formation of the disulfide bonds, the whole molecule were divided into peptides which contain only one disulfide bond according to the method of CHAPTER I. Figure VI-1A shows RP-HPLC patterns of the tryptic peptides derived from BrCN-cleaved wild-type lysozyme. From amino acid analysis, it was found that peak-a corresponded to the peptides Cys 6 ~ Homoserine 12 plus Gly 126 ~ Leu 129 which contained the disulfide bond, Cys 6 - Cys 127, that peak-b corresponded to the peptides Gly 22 ~ Lys 33 plus Cys 115 ~ Arg 121 which contained the disulfide bond, Cys 30 - Cys 115, and that peak-c corresponded to the peptides Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 which contained the disulfide bonds, Cys 64 - Cys 80 and Cys 76 - Cys 94. Figures VI-1B ~ VI-1F show the RP-HPLC patterns of the tryptic peptides derived from BrCN-treated mutant lysozymes where Trp 62 is mutated to Phe, His, Met, Asn, or Gly, respectively. From the amino acid composition of the peptides corresponding to peak-a and peak-b in these mutant lysozymes, it was found that disulfide bonds, Cys 6 - Cys 127 and Cys 30 - Cys 115, were correctly formed in all mutant lysozymes. From the amino acid composition, the peaks d - h in Figure VI-1B ~ VI-1F were found to correspond to the peptide Trp 62 ~ Arg 68 plus Asn 74 ~ Lys 96 whereas the retention times in these peaks were altered from that in peak-c due to each mutation.

Next, in order to analyze the formation of the disulfide bonds among Cys 64, Cys 76, Cys 80, and Cys 94, further digestion of the peptides in peak-c - h with α-chymotrypsin

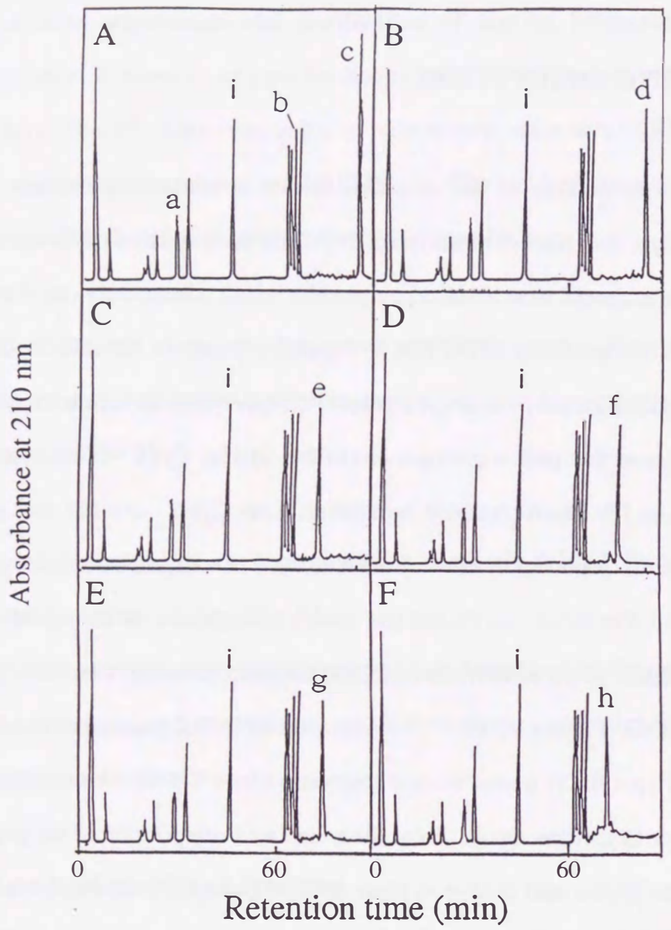

Figure VI-1 RP-HPLC of tryptic peptides derived from BrCN-treated lysozyme. A, Wild-type; B, W62F; C, W62H; D, W62M; E, W62N; F, W62G. The column (Wakosil C18, 4.6 mm x 250 mm) was eluted with a gradient of 50 ml of 1% acetonitrile and 50 ml of 50% acetonitrile both containing 0.1% HCl at a flow rate of 0.6 ml/min.

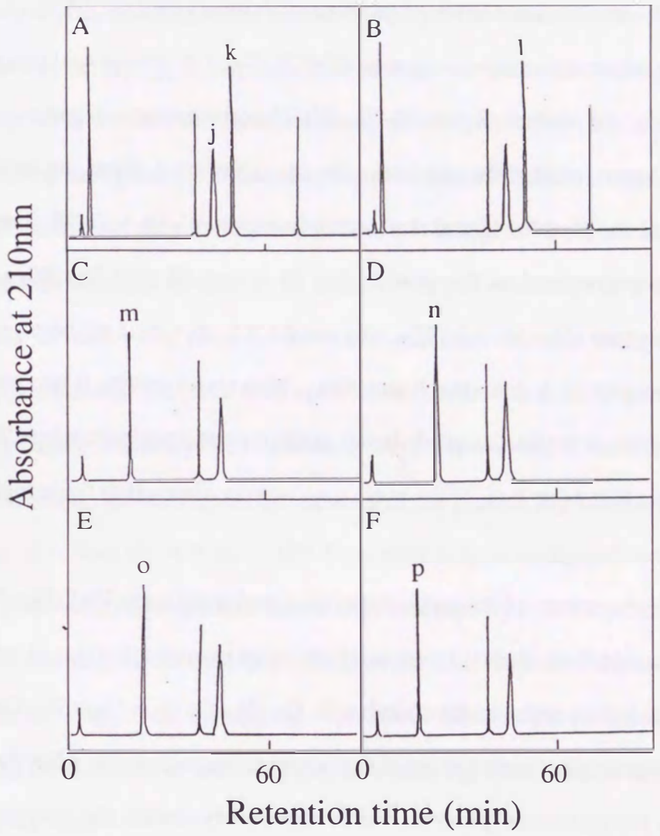

Figure VI-2 RP-HPLC of the digest of the peptide corresponding to Trp 62 ~ Arg 68 + Asn 74 ~ Lys 96 with α-chymotrypsin and prolylendopeptidase. A, W 62 F; B, W 62 H; C, W 62 M; D, W 62 N; E, W 62 G. The column (Wakosil C18, 4.6 mm x 250 mm) was eluted with a gradient of 40 ml of 1 % acetonitrile and 40 ml of 40% acetonitrile both containing 0.1% HCl at a flow rate of 0.6 ml/min.

and prolylendopeptidase were carried out according to the method of CHAPTER I. RP-HPLC patterns of the digested peptides are shown in Figure VI-2A ~ VI-2F. In order to examine the formation of the disulfide bonds, each peptide on RP-HPLC was isolated. Based on the results of CHAPTER I, peak-j in Figure VI-2A was identified to be the peptide Asn 74 ~ Pro 79 plus Asn 93 ~ Lys 96 (containing a cystine, Cys 76 - Cys 94). From the comparison of the retention time on RP-HPLC and the amino acid composition of the peptides in the peaks in Figure VI-2B ~ VI-2F corresponding to that of peak-j in Figure VI-2A were found to be identical. On the other hand, from the amino acid compositions of the peptides of peak-k ~ peak-o in Figure VI-2A ~ VI-2F, these peaks were found to correspond to the peptide Trp 62 ~ Arg 68 plus Cys 80 ~ Ala 82 (containing a cystine, Cys 64 - Cys 80) ,whereas the retention time of these peaks was altered from that of peak-k due to each mutation. From above results, it was concluded that the formations of the four disulfide bonds in the mutant lysozymes where Trp 62 is mutated to Phe, His, Met, Asn, or Gly were qualitatively identical to those in the wild-type lysozyme.

To compare the amount of the peaks, that of each peak-i in Figure VI-1 (Phe 34 ~ Arg 45) that contains no disulfide bond were employed as an internal standard. It was found that the peptide that contains the disulfide bonds, Cys 64 - Cys 80 or Cys 74 - Cys 96, in some Trp 62 mutant lysozymes was not always recovered quantitatively while the peaks a and b in each Trp 62 mutant lysozyme, which respectively contain the disulfide bonds, Cys 6 - Cys 127 or Cys 30 - Cys 115, were found to be quantitatively recovered. I set the ratio of the amount of peak-c to that of peak-i at 1. The ratios of the amount of peaks d - h to that of the respective peak-i are evaluated and shown in Table VI-1. The ratios in W 62 F lysozyme or W 62 H lysozyme, in which the residue is considerably bulky, were identical to that in the wild-type lysozyme. However, the ratio decreased with a decrease in the size of residue 62, and in W 62 G lysozyme, it was 0.8 of that of the wild-type lysozyme. On the other hand, the order of the ratio was not always consistent with that

of hydrophobicity of the residue at the position 62 since Met residue has higher hydrophobicity than His residue (Kyte and Doolittle, 1982).

In order to examine why the amount of the tryptic peptide including the residue 62 in several mutant lysozymes decreased, I prepared the mutant hen lysozyme where Cys 80 is mutated to Ala, resulting in the formation of cysteine residue in the molecule. RP-HPLC pattern of the tryptic peptides derived from the BrCN-treated mutant lysozyme where Cys 80 is mutated to Ala was considerably different from that of the wild-type lysozyme, indicating that the produced cysteine residue affected the formation of the other disulfide bond(s) and the pattern was similar to that in Figure VI-4B (data not shown). A similar pattern was also observed in the mutant lysozyme where Cys 76 is mutated to Ala (data not shown). Taniyama *et al.* have demonstrated that sulfhydryl-disulfide interchange reactions take place even under acidic conditions in the fragmentation of the mutant lysozyme where Cys 81 in human lysozyme is mutated to Ala (Taniyama *et al*, 1990). Therefore, the decrease in the recovery of the peptide Trp 62 ~ Arg 68 plus Asn 73 ~ Lys 96 in Met 62, Asn 62 or Gly 62 mutant may be explained by the following reason; since sulfhydryl-disulfide interchange reactions by cysteine occur in the tryptic peptides containing the disulfide bond, the peptides form various disulfide bonds and are hard to be recovered from the column. This result indicated the production of cysteine residues in the molecule of mutant lysozyme where Trp 62 is mutated to Met, Asn, and Gly, respectively.

Table VI-1 The ratios of amount of peak-d ~h to that of respective peak-i.

Peak d (W62F)	1.0
Peak e (W62H)	1.0
Peak f (W62M)	0.9
Peak g (W62N)	0.9
Peak h (W62G)	0.8

Analysis of W 62 G lysozyme using gel chromatography

As was shown above, a species with cysteine residues was found to be present in the molecule of mutant lysozyme where Trp 62 is mutated to Gly. If the species with cysteine residues in the molecule of mutant lysozyme is present, it should have larger hydrodynamic volume than the wild type lysozyme due to lacking the disulfide bond. Therefore, in order to confirm the presence of cysteine residues in the mutants, immediately after the W 62 G lysozyme was dissolved in 10% aqueous acetic acid, the protein solution was applied to the column of gel chromatography equipped to HPLC and eluted with 10% aqueous acetic acid at a flow rate of 0.5 ml/min. Two peaks appeared under these conditions (Figure VI-3A). The retention time of the species in the latter peak was identical to that of the authentic hen lysozyme. The arrow indicates the eluted position of the reduced-*S*-carboxymethylated lysozyme, which is eluted earlier than that of the authentic lysozyme. Therefore, the species in the former peak in Figure VI-3A is found to have a larger hydrodynamic volume than the intact lysozyme and a much smaller hydrodynamic volume than the denatured lysozyme. The area in the former peak was about 20% of the total area in the two peaks, which was consistent with the decreased amount of peak-h in Figure VI-1F (Table VI-1). When W 62 G lysozyme, which was kept in 10% aqueous acetic acid at room temperature for 5 days, was applied to the column, the area in the former peak decreased to less than 10% of the total area in the two peaks (Figure VI-3B). Therefore, it was found that the species in the former peak was an unstable one, which is consistent with the idea that the species in the former peak in Figure VI-3A includes cysteine residues in the molecule.

To examine the products further, I collected each peak material in Figure VI-3A and lyophilized. The lyophilized sample was dissolved in 1 M HCl containing 6.0 M Gdn-HCl and stirred in the presence of BrCN (See Materials and Methods). Although the RP-HPLC pattern of the tryptic peptides derived from the BrCN-treated derivative in the latter peak in Figure VI-3A was almost identical to that of the wild-type lysozyme (Figure VI-

4A), that derived from the former peak in Figure VI-3A was clearly different from that of the wild-type lysozyme (Figure VI-4B) but was similar to that of the mutant lysozyme where Cys 80 is mutated to Ala, indicating that the species contains cysteine residues in the molecule. From these results, it was concluded that the species containing cysteine residues in the molecule was partly present in the W 62 G lysozyme (or the W 62 N lysozyme and W 62 M lysozyme) secreted from yeast.

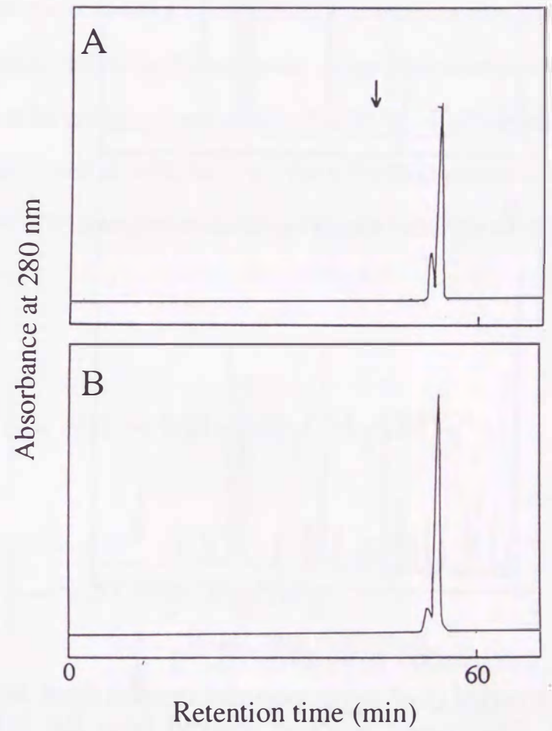

Figure VI-3 Gel filtration HPLC of W 62 G lysozyme. The protein solution was applied to the column A, immediately after W 62 G was dissolved in 10% aqueous acetic acid and B, after W 62 G had been kept in 10% aqueous acetic acid for 5 days at room temperature. The arrow indicates the eluted position of the reduced-*S*-carboxymethylated lysozyme. The column was eluted with 10% aqueous acetic acid at a flow rate of 0.5 ml/min.

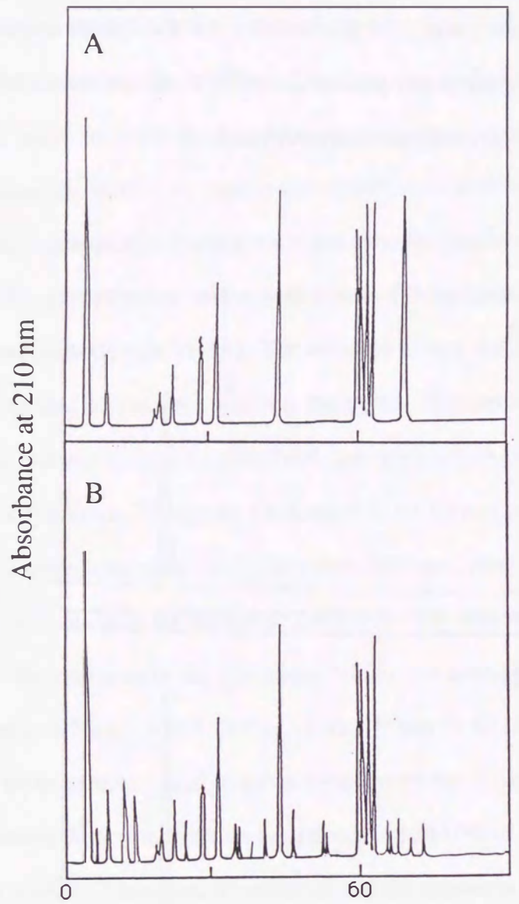

Figure VI-4 RP-HPLC of trypic peptides derived from BrCN-treated W 62 G. A, The tryptic peptides derived from the BrCN-treated derivative in the latter peak in Figure VI-3A. B, The tryptic peptides derived from the BrCN-treated derivative in the former peak in Figure VI-3A. The column (Wakosil C18, 4.6 mm x 250mm) was eluted with a gradient of 50 ml of 1% acetonitrile and 50 ml of 50% acetonitrile both containing 0.1% HCl at a flow rate of 0.6 ml/min.

Trapping of cysteine residues present in the Trp 62 mutant lysozyme

In order to confirm the formation of the cysteine residues further, W 62 G lysozyme, which has the highest population of the species with the cysteine residue, was dissolved in Tris-HCl buffer containing 1 mM AEMTS which has higher reactivity than monoiode acetate (Rothewarf and Scheraga, 1991). In this procedure, if there is a cysteine residue in the molecule, the cysteine residue should be modified with AEMTS (Figure VI-5). After incubation at 40°C for 10 min, however, no amount of cysteine in W 62 G lysozyme could the modified with AEMTS. Then, W 62 G lysozyme was dissolved in 8.0 M urea solution containing 1 mM AEMTS in order to accelerate the access of the reagent to cysteine residue in the molecule. After incubation at 40°C for 10 min, the concentration of the urea solution was diluted to 2.0 M. TPCK-trypsin was added to the solution and incubated at 40°C for 2 h. The RP-HPLC pattern of the tryptic peptide derived from W 62 G lysozyme is shown in Figure VI-6B. The RP-HPLC pattern of the

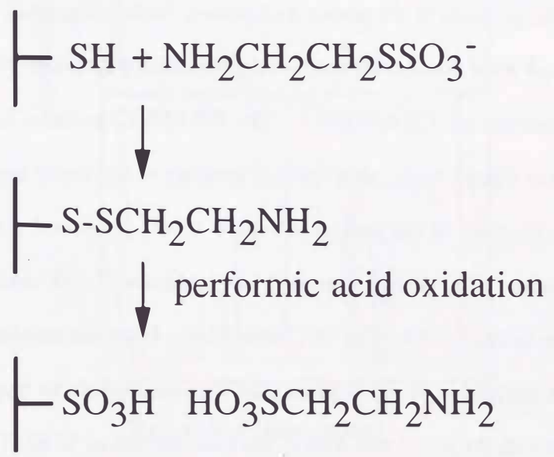

Figure VI-5 Reaction of AEMTS and the conversion of aminoethylthiol attached to the cysteine residue to taurine.

tryptic peptide derived from wild-type lysozyme is also shown in Figure VI-6A. There was no peak except for undigested wild-type lysozyme (peak-I) in Figure VI-6A. On the other hand, partially digested peaks appeared except for undigested W 62 G lysozyme (peak-II) in Figure VI-6B. After incubation of the protein solution for 12h at 40°C, RP-HPLC pattern of respective tryptic peptides did not change. The digested amount in W 62 G lysozyme was estimated to be 20% of total amount from comparison of amount of peak-II after digestion with that before digestion (data not shown). Figure VI-6C shows the enlarged pattern of Figure VI-6B.

The amino acid composition of the tryptic peptides in Figure VI-6C were analyzed after acid hydrolysis of the peptides which has been treated by performic acid oxidation. By a series of treatment, aminoethylthiol attached to the cysteine residue is converted to taurine (Figure VI-5) which can be detected by amino acid analysis. The peaks a ~ d in Figure VI-6C have contained the disulfide bond(s). The amino acid composition of these peaks are shown in Table VI-2. Taurine was detected in the peak-c, but it was not in the other peaks. To investigate the formation of the disulfide bonds among Cys 64, Cys 76, Cys 80, and Cys 94 in the peptides of the peak-c and peak-d, further digestion of the peptide of peak-c or peak-d with α-chymotrypsin and prolylendopeptidase was carried out according to the method of CHAPTER I. The RP-HPLC patterns of the digested peptides are shown in Figure VI-7. As a result of analysis of the amino acid composition of these peaks, the peptides of the peak-d in Figure VI-6C possessed correct disulfide bonds, but the peptides of the peak-c possessed two cysteines, Cys 80 and Cys 94, and a non-native disulfide bond, Cys 64 - Cys 76 (Table VI-3). From the results of Figure VI-6, 20% of the total amount in W 62 G lysozyme was susceptible to tryptic digestion. This was consistent with the result that 20% of the total amount of W 62 G lysozyme had a larger hydrodynamic volume on gel chromatography. Therefore, the species which was susceptible to tryptic digestion in W 62 G lysozyme may be considered to lack at least one disulfide bond. Indeed, I could detect the peptide of peak-c in Figure VI-6C

which had two cysteines, Cys 80 and Cys 94, and a non-native cystine, Cys 64 - Cys 76. However, I also detected the peptide of peak-d in Figure VI-6C which had correct cystines, Cys 64 - Cys 80 and Cys 76 - Cys 94, while the other cystines, Cys 6 - Cys 127 and Cys 30 - Cys 115, correctly formed in the species. It is strange that the species with the correct disulfide bonds could have been susceptible to tryptic digestion.

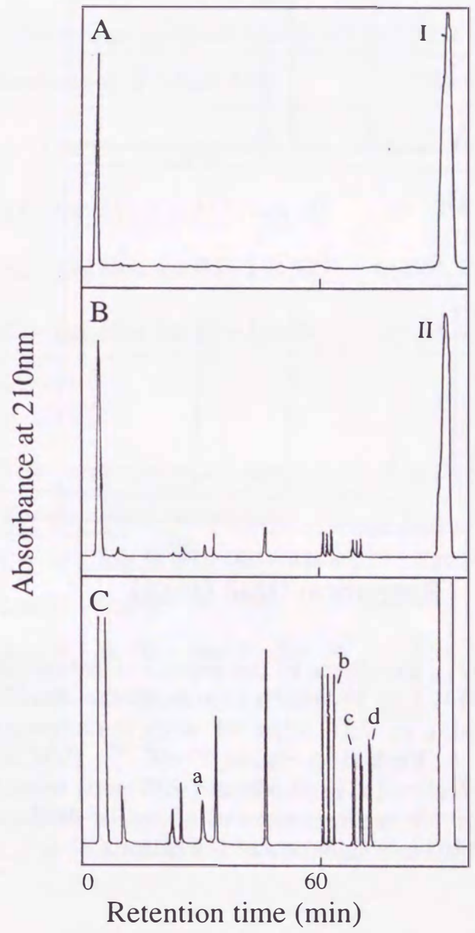

Figure VI-6 RP-HPLC of trypic peptides derived from A, wild-type and B, W 62 G in AEMTS. C, the magnification of B. The column (Wacosil C18, 4.6 mm x 250 mm) was eluted with a gradient of 50ml of 1% acetonitrile and 50 ml of 50% acetonitrile both containing 0.1 % HCl at a flow rate of 0.6 ml/min.

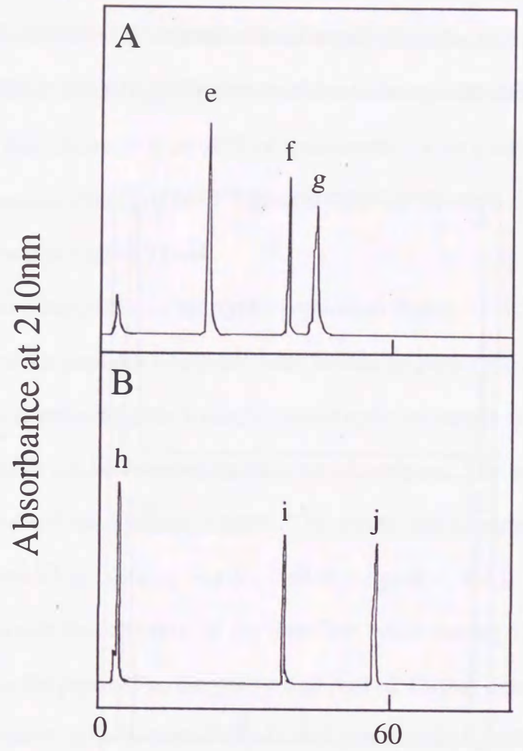

Figure VI-7 RP-HPLC of the digest of the peptide corresponding to Trp 62 ~ Arg 68 + Asn 74 ~ Lys 96 which contained the disulfide bonds, Cys 64 - Cys 80 and Cys 76 - Cys 94 with α-chymotrypsin and prolylendopeptidase. A, Peak d in Figure VI-6C. B, Peak c in Figure VI-6C. The column (Wakosil C18, 4.6 mm x 250 mm) 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.

Table VI-2 The composition of amino acid of peak-a ~ d in Figure 6C.

Peak	Composition of Amino Acid	Cystin or Cysteine	Taurine
a	Cys 6 ~ Lys 13 + Gly 126 ~ Arg 128	Cys 6 - Cys 127	0
b	Gly 22 ~ Lys 33 + Cys 115 - Lys 116	Cys 30 - Cys 115	0
c	Gly 62 ~ Arg 68 + Asn 74 ~ Lys 96	Cys 64, Cys 76, Cys 80, Cys 90	2
d	Gly 62 ~ Arg 68 + Asn 74 ~ Lys 96	Cys 64, Cys 76, Cys 80, Cys 90	0

Table VI-3 The composition of amino acid of peak-e ~ j in Figure VI-7.

Peak	Composition of Amino acid	Cysteine or Cysteine	Taurine
e	Gly 62 ~ Arg 68 + Cys 80 ~ Ala 82	Cys 64 - Cys 80	0
f	Leu 83 ~ Val 92	-	0
g	Asn 74 ~ Ile 78 + Asn 93 ~ Lys 96	Cys 76 - Cys 90	0
h	Cys 80 ~ Ala 82, Asn 93 ~ Lys 96*	Cys 80, Cys 90	2
i	Leu 83 ~ Val 92	-	0
j	Gly 62 ~ Arg 68 + Asn 74 ~ Ile 78	Cys 64 - Cys 76	0

*Two peptides, Cys 80 ~ Ala 82 and Asn 93 ~ Lys 96, in peak-h were overlapped.

The phenomenon obtained here may be able to be explained as shown in Figure VI-8. Namely, 20% of the total amount of W 62 G lysozyme may be the species which had two cysteines, Cys 80 and Cys 94, and a non-native cystine, Cys 64 - Cys 76, while the other cystines, Cys 6 - Cys 127 and Cys 30 - Cys 115, correctly formed. During trapping cysteine residues in the molecule by AEMTS, the structure of lysozyme may become looser due to the addition of urea, resulting in a partial recombination of the cysteine - cystine of the species to occur at cysteine 80 and cystine, Cys 64 - Cys 76, and the formation of correct disulfide bonds, Cys 64 - Cys 80 and Cys 76 - Cys 94, whereas cysteine 80 and 94 were trapped by AEMTS in the other species . As a result, the correct disulfide bonds also formed and gave the formation of the peptide of peak-d in Figure VI-6C. This procedure of the formation of correct disulfide bonds in lysozyme was not novel, which was reported in the previous literature (Kikuchi *et al*, 1988). Moreover, our above explanation was supported by the following additional examination. First, when I employed the less amount of trapping reagent than above, the peptide in peak-c decreased and the resulting peptide in peak-d (correct formation of disulfide bonds) increased, indicating that the amount of unreacted Cys 80 increased. Second, Cys 94 was easier to react with the trapping reagent than Cys 80 due to its higher accessibility. Third, the partial reduction and oxidation of the peptide in peak-c gave the formation of the peptide in peak-d, which means that the intramolecular exchange reaction at Cys 80 and cystine, Cys 64 - Cys 76 occurs even in tryptic peptide. Additionally, the idea that the species amounted to 20% of the total amount of W 62 G lysozyme had two cysteines, Cys 80 and Cys 94 and a non-native cystine, Cys 64 - Cys 76 was also consistent with the result that tryptic peptide containing Cys 64, Cys 76, Cys 80 and Cys 94 had decreased by 20% on RP-HPLC. Therefore, from above results, I elucidated the formation of cysteine residues in W 62 G lysozyme expressed from yeast.

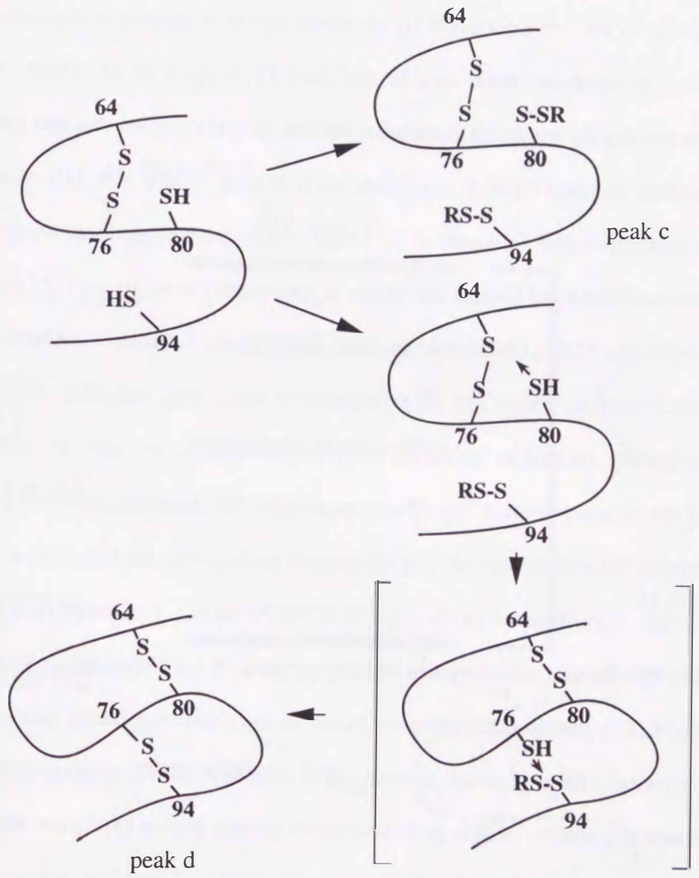

Figure VI-8 Trapping of cysteine residues present in W62G.

Meaning of the finding of the species with the cysteine residue in the mutant hen lysozyme at the position 62

Using yeast, *Saccharomyces cerevisiae*, chicken type lysozymes such as hen and human lysozyme can be produced in native form. It is generally believed that one gene directs the synthesis of one protein. However, there were a few reports that some species, which are involved in cysteine residue, were produced in the culture fluid from *S. cerevisiae* in case of mutant human lysozyme where cysteine residue is mutated to the other amino acid residues (such as Ala and Ser) (Taniyama *et al*, 1990; 1988). Moreover, except for the mutation at cysteine residue, it was reported that there were at least four species secreted from *S. cerevisiae* in a mutant where Val 110 of human lysozyme is mutated to Pro (Kikuchi *et al*, 1988). The present result that there were plural species in culture fluid from *S. cerevisiae* is also worthy to note.

In the present CHAPTER, I have demonstrated that cysteine residues were formed in a part of mutant lysozyme where Trp 62 is mutated to Met, Asn, and Gly. This result could be intimately related to the result of lysozyme folding *in vitro* in which the oxidation of the reduced peptide Trp 63 ~ Homoserine 105 including Cys 64, Cys 76, Cys 80, and Cys 94 and devoid of Trp 62 yielded exclusively the non-native cystine residues, Cys 64 - Cys 76 and Cys 80 - Cys 94 (CHAPTER I). This result may indicate the possibility that the non-native cystine residue forms in W 62 G lysozyme, where Trp 62 is mutated to Gly, because the mutation removes the restriction on the movement of Trp 63 in the reduced form. Indeed, the non-native cystine residue corresponding to Cys 64 - Cys 76 was reported to be able to be formed in mutant human lysozyme where Cys 81 and Cys 95 were mutated to Ala (Taniyama *et al*, 1990). Therefore, when the non-native cystine residue, Cys 64 - Cys 76, is formed in W 62 M, W 62 N, and W 62 G lysozyme, the remaining Cys 80 and Cys 94 might not form a disulfide bond due to steric hindrance, resulting in the production of cysteine residues while the other correct cystines Cys 30 - Cys 115 and Cys 6 - Cys 127 is formed. Namely, a bulky residue such as Trp,

Phe, or His at the position 62 of hen lysozyme would lead to the correct formation of disulfide bonds. However, Met, Asn, or Gly, which are less bulky than Trp, Phe, or His may lead to partial formation of the species containing non-native disulfide bonds and cysteine residues. The amount of the species containing cysteine residues may be reflected in the extent of the interaction to form the non-native disulfide bond, Cys-64 - Cys-76, in the nascent state (Figure VI-9).

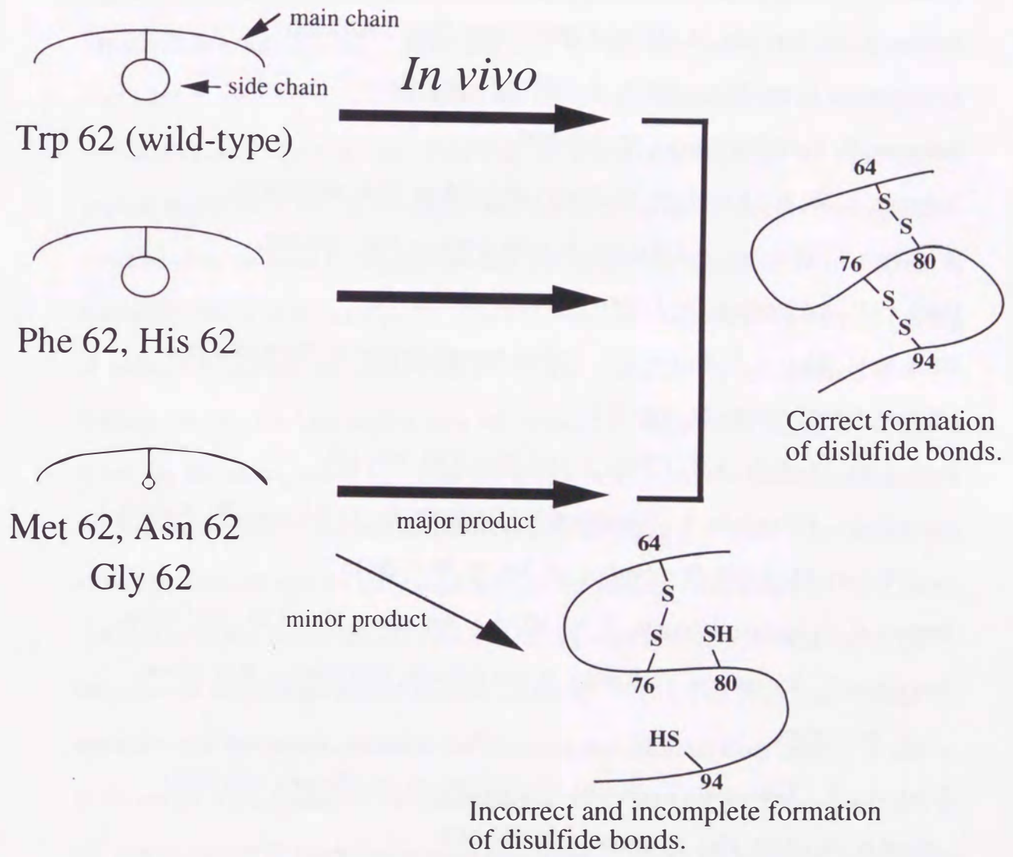

Figure VI-9 The formation of disulfide bonds in the wild-type lysozyme and the mutant lysozymes secreted from yeast.

From these results, in the present case, the information of the folding process of hen lysozyme *in vitro* was found to be consistent with that *in vivo*. However, further analyses would have to be required in order to elucidate that the entire folding process of hen lysozyme *in vitro* was identical to that *in vivo*. Anyway, the present results set forth the notion that the conformation of mutant proteins secreted from yeast should be analyzed more carefully.

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CONCLUSION

In this thesis, I analyzed the folding process of hen lysozyme from reduced form, especially on the early events. In CHAPTER I, I developed the method of qualitative and quantitative analysis of four disulfide bond formations in hen lysozyme. This method, enables us to examine the formation of the disulfide bonding in oxidative folding of lysozyme. In CHAPTER II, I found that the peptide fragment 59 ~ 105 of lysozyme formed the correct disulfide bond from reduced form without the help of the other peptide region. Moreover, Trp 62 and Trp 63 was deeply involved in the correct formation of two disulfide bonds (Cys 64 - Cys 80 and Cys 76 - Cys 94), and it was shown that the interaction of these Trp residues with α-helix 88 ~ 98 was important for the folding of the peptide fragment 59 ~ 105. In CHAPTER III, it was concluded that the formation of peptide region 59 ~ 105 in lysozyme was cue in the folding process leading active structure of the reduced lysozyme, and that Trp 62 and the formation of two disulfide bonds (Cys 64 - Cys 80 and Cys 76 - Cys 94) were important for early stage of folding of reduced lysozyme. In CHAPTER IV, I have found that by introduction of Ca^{2+} binding site into the loop region 82 ~ 91 included in the peptide region 59 ~ 105 of lysozyme, the folding rate of lysozyme was accelerated. Therefore, it was suggested that the formation of region connecting between α–domain and β-domain in lysozyme was closely participated with the early stage of lysozyme folding. In CHAPTER V, I found that CaB lysozyme was efficiently folded in the presence of high concentration of Ca^{2+}. Moreover, it was concluded that the loop region 82 ~ 91 in CaB lysozyme formed the some structure that loosely bound to Ca^{2+}, which was different from random structure, in the earlier folding stage of the reduced form. In CHAPTER VI, I found that in Met 62, Asn 62, or Gly 62 lysozyme secreted from yeast, in which the residue at the position 62 was less bulky, contained the species having two cysteines, Cys 80 and Cys 94, and a non-native cystine, Cys 64 - Cys 76. Therefore, it may be suggested that the bulkiness at

the position 62 may be required for the formation of correct disulfide bonds. Moreover, it may be considered that the folding in the early stage of lysozyme *in vivo* was resemble to that *in vitro*.

In this thesis, I found the important interaction, key residue, and core region in the early stage of the folding process of lysozyme. These may be only one of milestones in the elucidation of the general rule of protein folding. These information coupled with the prospective research such as elucidation of the folding mechanism of the core region may help to design the functional analysis of proteins determined by human genome or to conduct de novo design of functional proteins, which participate in pharmaceutical sciences.

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