Kinetically Trapped Structure in the Renaturation of Reduced Oxindolealanine 62 Lysozyme

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ABSTRACT: The refolded products of reduced native lysozyme and reduced OX62 lysozyme, in which Trp62 is converted to oxindolealanine (OX62) during the renaturation of sulfhydryl−disulfide interchange reactions at pH 8 and 37 °C, were investigated. On gel-chromatography eluted with 10% aqueous acetic acid containing 4 M urea, two peaks appeared in the refolded product of reduced OX62 lysozyme while a single peak appeared in the refolded product of reduced native lysozyme. From the analyses of the activity and primary and the tertiary structures of the derivative, the structure of the derivative from reduced native lysozyme was confirmed to be identical to that of the untreated one. On the other hand, the refolded product from reduced OX62 lysozyme had the same primary structure but a different tertiary structure compared to the untreated one. The tertiary structure of the refolded product from the reduced OX62 lysozyme was changed to that of the untreated one by the denaturation−renaturation treatment under nonreduced conditions. However, the refolded species was barely changed to that of the untreated one by incubation under physiological conditions. Therefore, the refolded product from reduced OX62 lysozyme was suggested to be a metastable and kinetically trapped product in the renaturation process of reduced OX62 lysozyme. In addition, an interaction involving the folding process of reduced lysozyme was discussed on the basis of the NMR analyses of the metastable structure.

Genetic engineering techniques have enabled us to obtain a large amount of desired polypeptide chains by using the *Escherichia coli* expression system. Because the desired proteins are often expressed as inclusion bodies in this system, we must refold them in order to obtain functional proteins. Proteins can be refolded to their native structure in which they can function (Anfinsen, 1973); however, it has been shown that the folding pathway is altered by the replacement of amino acid residues (Zhang & Goldenberg, 1993). In addition, it has also been shown that the folding rates (Ueda *et al.*, 1994) or the yields (Imoto *et al.*, 1987; Ueda *et al.*, 1990) in the folding of reduced proteins were changed by the modification of amino acid residues. Under these circumstances, we question if the final folding structure of a protein from the unfolded state is identical to the structure of an untreated one. In a chemically modified protein, a drastic conformational change would not occur in the folded state because most chemical modifications are introduced in the folded state of a protein. Therefore, a chemically modified protein should be suitable to investigate whether the final folded structure from the unfolded state is affected by the modification.

Selective chemical modification of hen egg white lysozyme have been vigorously carried out (Hayashi *et al.*, 1965; Imoto *et al.*, 1973, 1987). Moreover, the folding of lysozyme has also been investigated (Anderson & Wetlaufer, 1976; Miranker *et al.*, 1991; Radford *et al.*, 1992). Therefore, lysozyme is one of the more suitable proteins for the present purpose. In the refolding of reduced OX62 lysozyme[1] by the sulfhydryl−disulfide interchange reactions, its final folding yield was lower than that of native and oxindolealanine 108 (OX108) lysozyme which has the same modified structure (Ueda *et al.*, 1990). In this paper, we demonstrated that a kinetically controlled non-native structure could be trapped in the refolding of reduced OX62 lysozyme.

MATERIALS AND METHODS

Materials. Recrystallized (five times) hen egg white lysozyme was donated by the QP Company (Tokyo). Sephadex G-75 (medium) was purchased from Pharmacia. Columns of SW-3000 (7.5 × 600 mm), Wakosil 5C18-200 (4.6 × 250 mm), YMC Pack A-212 C8 (6 × 150 mm), and Cosmosil C4 (4.6 × 150 mm) were obtained from Tosoh (Tokyo), Wako Pure Chemical Industries, Ltd., Yamamura Chemical Lab (Tokyo), and Nacalai Tesque Inc. (Kyoto), respectively. Both TPCK-trypsin and α-chymotrypsin were products of Worthington. Prolylendopeptidase was purchased from Seikagaku Kogyo Ltd. *Micrococcus luteus*, a substrate of lysozyme, was purchased from Sigma. D_2O, DCl, and NaOD were products of C.E.A. Lysozymes OX62 (Hayashi *et al.*, 1965) and OX108 (Imoto *et al.*, 1973) were prepared respectively, as previously described. Other chemicals were purchased from Nacalai Tesque Inc. or Wako Pure Chemical Industries, Ltd. (Tokyo).

Gel Chromatography of Lysozyme. A 50 μg amount of native lysozyme was applied to columns of TSK-gel SW-

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[1] Abbreviations: CD, circular dichroism; BrCN, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; Gdn-HCl, guanidine hydrochloride; OX62 lysozyme, oxindolealanine 62 lysozyme; OX108 lysozyme, oxindolealanine 108 lysozyme; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; TPCK-trypsin, L-1-(*p*-tosylamino)-2-phenylethyl chloromethyl ketone-treated trypsin.

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3000 (7.5 mm × 600 mm) which were equilibrated with 10% aqueous acetic acid containing 0, 2, 4, and 5 M urea, respectively, at a flow rate of 0.3 mL/min.

Acid Urea Gel Chromatography of the Refolding of Reduced Lysozymes. Renaturation of lysozyme was carried out according to Ueda *et al.* (1990) with a slight modification. Briefly, lysozymes (2 mg) were dissolved in 1 mL of 8 M urea solution [0.584 M Tris-HCl buffer (pH 8.6) containing 8.125 M urea and 5.37 mM EDTA] and reduced with fresh 2-mercaptoethanol (12.5 μL) at 40 °C for 90 min under a nitrogen atmosphere. Each solution (200 μL) was rapidly diluted with 19.8 mL of 0.2 M Tris-HCl buffer (pH 8) containing 1 mM EDTA and 0.66 mM oxidized glutathione that were preincubated at 40 °C for 30 min. After the pH of each solution was lowered to 5.5, 125 mg of *N*-ethylmaleimide was added to each and the resulting mixtures were stirred for 30 min at room temperature. The pH of each solution was then lowered to 3 by adding acetic acid to stop the reaction. The solution was immediately applied to the column of Sephadex G-75 (3 cm × 180 cm) which was equilibrated with 10% aqueous acetic acid containing 4 M urea. The protein elution was monitored by measuring the absorbance at 280 nm. The collected protein fraction was dialyzed against distilled water. For further purification, the dialysate was applied to the column of Cosmosil C4 equipped with HPLC that was equilibrated with 1% acetonitrile containing 1% acetic acid at a flow rate of 1 mL/min. The column was eluted with 50% acetonitrile containing 1% acetic acid. The eluted protein fraction was lyophilized.

BrCN Cleavage of Lysozymes. Lysozyme, 3 mg, was dissolved in 1 mL of 0.1 M HCl containing 6 M Gdn-HCl. A 20 mg amount of BrCN was added to the solution as a solid, and the solution was stirred for 16 h at room temperature to cleave the peptide bond at Met12 and Met105. The cleaved lysozyme was applied to the column of Cosmosil C4 equipped with HPLC that had been equilibrated with 1% acetonitrile containing 1% acetic acid to remove the salts. The column was eluted with 60% acetonitrile containing 1% acetic acid at a flow rate of 1 mL/min. The eluted protein fraction was lyophilized.

Protease Digestion. A 2 mg amount of BrCN-cleaved lysozyme was dissolved in 5 mL of 0.1 M phosphate buffer (pH 6.5); 40 μg of TPCK-trypsin was added to the solution and incubated at 40 °C for 4 h. To analyze the formation of cystines (Cys 64, Cys 76, Cys80, and Cys94), 50 μg of tryptic peptide, which contained the above four cystines and which had been separated on RP-HPLC, was dissolved in 500 μL of 0.1 M phosphate buffer at pH 6.5. α-Chymotrypsin, 2 μg, was added to the solution, which was incubated at 40 °C for 2 h, and then 0.01 unit of prolylendopeptidase was added, and the resulting solution was incubated at 40 °C for 10 min.

A 500 μg amount of BrCN-cleaved lysozyme was dissolved in 2 mL of 0.05 M Tris-HCl buffer at pH 6. Lysylendopeptidase, 5 μg, was added to the solution, which was incubated at 35 °C for 4 h.

Analytical Methods. RP-HPLC of the peptides was carried out by monitoring the eluted peptide at 210 and 280 nm according to the method of Ueda *et al.* (1991). Peroxidation of the peptides was carried out according to the method of Harris (1967). Amino acid analysis was performed on a Hitachi L-8500 amino acid analyzer after hydrolysis of a sample in 6 N HCl at 110 °C for 20 h. The enzymatic activities of the lysozymes against *M. luteus* were measured turbidimetrically at 450 nm at pH 7.0. To a 3 mL suspension of *M. luteus* in 0.05 M phosphate buffer at pH 7.0 was added 100 μL of lysozyme solution, and the decrease in the turbidity was monitored with a Hitachi 150−20 spectrophotometer equipped with a thermostatically controlled cell holder. SDS−PAGE was carried out according to the method of Laemmli (1970).

Circular Dichroism (CD) Spectra. Circular dichroism spectra (190−300 nm) were measured with a JEOL-720 spectrophotometer at 23 °C using a 10 μM lysozyme in 0.02 M phosphate buffer (pH 8). The cell used had a 0.1 cm path length.

Two-Dimensional ^{1}H-NMR. ^{1}H-NMR spectra were recorded with a Varian Unity Plus 600 MHz NMR spectrometer. The probe temperature was calibrated with ethylene glycol. Dioxane was employed as the internal standard (3.743 ppm). The pH values were the pH meter readings without adjustment for isotope effects (Bundi & Wüthrich, 1979). Phase-sensitive double-quantum-filtered COSY (Bodenhauser *et al.*, 1984) and phase-sensitive NOESY (Marion & Wüthrich, 1983) experiments were carried out at 600 MHz using standard procedures. Typically, 32 transients were recorded for each of 512 increments. NOESY spectra were acquired with mixing times of 150 ms. A digital resolution of 2.4 Hz/point in both dimensions was used for the COSY and NOESY.

RESULTS AND DISCUSSION

Size Exclusion Chromatography of Lysozymes with Aqueous Acetic Acid Containing Urea. To separate the lysozyme species after the renaturation of the reduced form, electrophoretic analysis was employed using the low discontinuous electrophoresis system in the presence of concentrated urea on a polyacrylamide slab gel (Dubois *et al.*, 1982; Fisher *et al.*, 1992). However, because this system can develop only small amounts of sample, it is inadequate to analyze the renaturated species whereas size exclusion chromatography is an appropriate method to separate conformational species such as the folded or the unfolded state of a protein (Tanford *et al.*, 1967; Uversky, 1993). Therefore, we examined the stability of native lysozyme in 10% aqueous acetic acid against urea in order to find out the condition at which native protein is denatured. When native lysozyme was analyzed on gel chromatography, TSK-gel SW-3000, which was eluted with 10% aqueous acetic acid containing less than 4 M urea at 20 °C, a single peak appeared while the peak became broad with the increase in the urea concentration. In the elution condition where 10% acetic acid contains more than 5 M urea, the shoulder peak appeared. Thus, we employed the condition of 10% acetic acid containing 4 M urea at 20 °C where native lysozyme was barely separated.

From our previous results, 80% of the reduced lysozyme was refolded to an active form at a concentration of 100 μg/mL (Maeda *et al.*, 1994). Therefore, the refolding procedures of reduced lysozymes were carried out at a concentration of 100 μg/mL. After the reduced native lysozyme was refolded by the catalysis reaction of both 2-mercaptoethanol provided by the reduction mixture and oxidized glutathione, it was reacted with *N*-ethylmaleimide (see Materials and Methods). The reaction mixture was

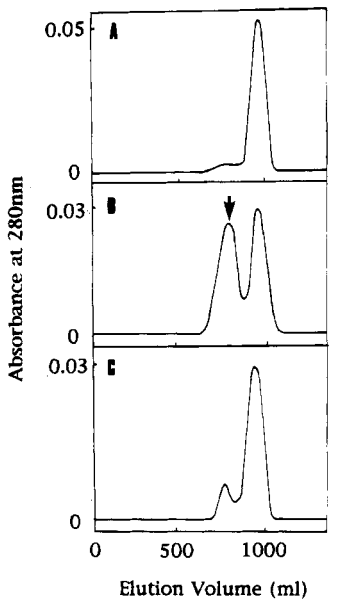

FIGURE 1: Gel chromatography of the refolded product from reduced native lysozyme (A), OX62 lysozyme (B), and OX108 lysozyme (C). The column (Sephadex G-75, 3 × 180 cm) was eluted with 10% aqueous acetic acid containing 4 M urea. The arrow indicates the elution position of BrCN-cleaved lysozyme.

immediately applied to the column of Sephadex G-75 (3 cm × 180 cm) which was equilibrated with 10% acetic acid containing 4 M urea. The elution pattern is shown in Figure 1A. The elution position of the major peak (*ca.* 95% yield) was the same as that of an untreated one. The fraction was collected and dialyzed against distilled water. Then, the dialysate was purified by RP-HPLC (see Materials and Methods). The collected protein fraction was lyophilized. This fraction consisted of monomeric lysozyme from the analysis of nonreduced SDS−PAGE. The lytic activity of the refolded sample against *M. luteus* was identical to that of the untreated lysozyme.

On the other hand, in our previous report (Ueda *et al.*, 1990), the final folding yield of reduced OX62 lysozyme was shown to decrease relative to the yields of both native lysozyme and OX108 lysozyme whose type of modification is identical to OX62 lysozyme. This result indicates that the modification of Trp62 to oxindolealanine somewhat affected the folding of reduced lysozyme. Therefore, acid urea gel chromatography of the refolded species from reduced OX62 lysozyme was carried out (Figure 1B). Two major peaks appeared while only one major peak appeared in the case of OX108 lysozyme (Figure 1C). The derivative in the latter peak had the same elution volume as the native and untreated OX 62 lysozyme. Both peaks were further purified by use of RP-HPLC and lyophilized to examine their structure (see Materials and Methods). We analyzed the derivative in the latter peak first because the former peak resulted from several refolded species with incomplete formation of disulfide bonds (described later) and because the latter peak resulted from a single refolded species (described below). The derivative in the latter peak, which is named the refolded OX62 lysozyme, consisted of monomer of lysozyme based on analysis of nonreduced SDS−PAGE. However, the lytic activity of the refolded OX62 lysozyme against *M. luteus* was 1% relative to that of native lysozyme while the lytic activity of untreated OX62 lysozyme was 20%. This result indicates that the structure of the active

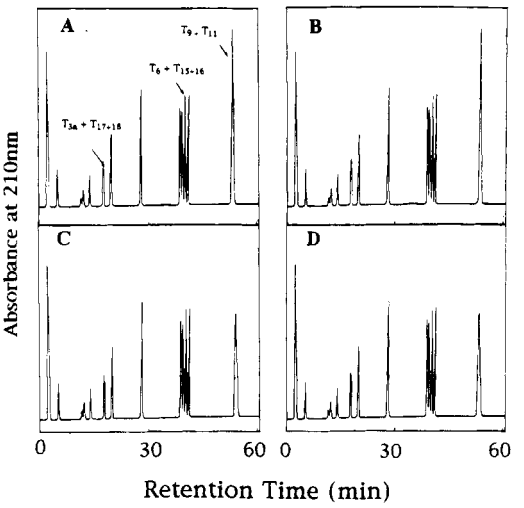

FIGURE 2: RP-HPLC of tryptic peptides derived from the BrCN-cleaved untreated native lysozyme (A), the refolded native lysozyme (B), the untreated OX62 lysozyme (C), and the refolded OX62 lysozyme (D). The column (YMC-A212 C8, 6 × 150 mm) was eluted with a gradient of 50 mL of 1% acetonitrile and 50 mL of 50% acetonitrile both containing 0.1% HCl at a flow rate of 0.8 mL/min. Assignments of peaks including cystine are as follows: T_{3a}, Cys6−Homoserine12; T_6, Gly22−Lys33; T_9, Trp62−Arg68; T_{11}, Asn74−Lys96; T_{15+16}, Cys115−Arg125; T_{17+18}, Gly126−Leu129.

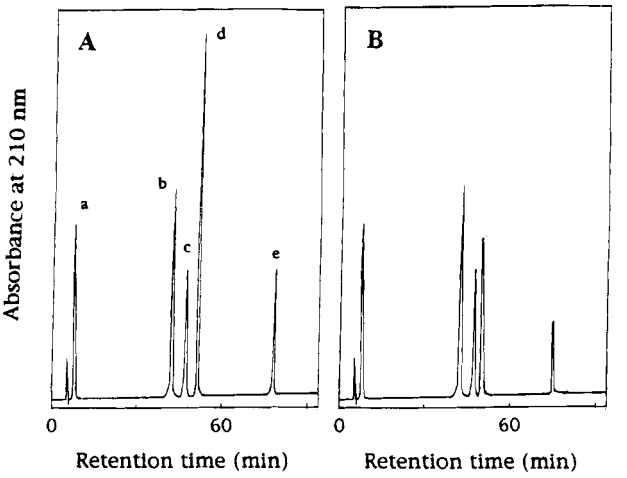

FIGURE 3: RP-HPLC of protease digest of the peptide corresponded to T_9+T_{11} in Figure 2B (A) and in Figure 2D (B) by both α-chymotrypsin and prolylendopeptidase at pH 6.5 and 40 °C. The column (Wakosil 5C18−200, 4.6 × 250 mm) was eluted with a gradient of 40 mL of 1% acetonitrile and 40 mL of 40% acetonitrile, both containing 0.1% HCl, at a flow rate of 0.6 mL/min.

site in the refolded OX62 lysozyme was different from that in untreated OX62 lysozyme. Therefore, we examined the difference in the structure between the untreated and the refolded OX62 lysozyme in comparison to that of native lysozyme below.

Primary Structures of the Refolded Lysozymes. In order to examine the primary structure of the refolded protein, the refolded protein that was treated with BrCN was dissolved in 0.1 M phosphate (pH 6.5), where the exchange of the disulfide bond was restrained, and incubated with TPCK-trypsin at 40 °C for 4 h (see Materials and Methods). The elution pattern of the tryptic peptides obtained from the refolded protein is shown in Figure 2B. For comparison, the pattern of native lysozyme is shown in Figure 2A in which the assignments of the peptide, including cystines, are

Table 1: Amino Acid Composition of Peptides after Acid Hydrolysis

	peptide[a]									
	a		b		c		d		e	
amino acid	calcd	theory	calcd	theory	calcd	theory	calcd	theory	calcd	theory
Asp	1.7	2	2.0	2	2.0	2	2.1	2	–	–
Thr	–	–	0.8	1	–	–	–	–	–	–
Ser	1.1	1	2.7	3	–	–	0.8	1	–	–
Gly	1.3	1	–	–	–	–	1.2	1	–	–
Ala	1	1	1	1	1	1	1	1	–	–
Val	–	–	1.0	1	–	–	–	–	–	–
Ile	–	–	0.9	1	0.7	1	–	–	–	–
Leu	–	–	1.0	1	0.9	1	–	–	–	–
Lys	–	–	–	–	0.9	1	–	–	–	–
Arg	0.7	1	–	–	–	–	0.8	1	–	–
Pro	–	–	–	–	1.0	1	–	–	–	–
Cys–SO_3H	2.0	2	–	–	2.0	2	2.0	2	–	–
Trp	–	–	–	–	–	–	+	+	+	+
assigned residue no.	64–68 | 80–82		84–92		74–76–79 | 93–94–96		62–64–68 | 80–82		62–63	

[a] In Figure 3A.

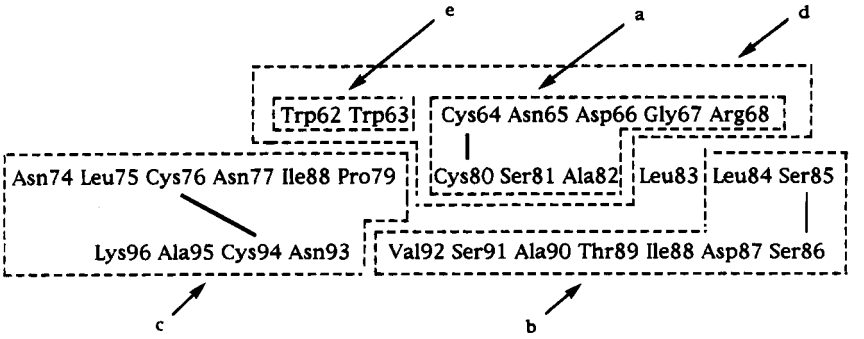

FIGURE 4: Primary structure of T_9+T_{11}. Thick lines indicate the disulfide bond. The assignment of each peptide in Figure 3A was carried out by enclosing each with a dotted line.

also shown. There were no differences in these patterns. To examine the disulfide pair in the peptide with the same retention time as the T_9+T_{11} peptide, the tryptic peptide was separated and lyophilized. The peptide was redissolved in 0.1 M phosphate buffer at pH 6.5 and incubated with α-chymotrypsin and then with prolylendopeptidase (See Materials and Methods). In Figure 3A, the elution pattern of the digest on RP-HPLC is shown. The amino acid compositions of the peptides that had been oxidized by performic acid were analyzed after acidic hydrolysis (Table 1). In Figure 4, the amino acid sequences in T_9 and T_{11} are shown. Because peptides d and e could be detected at 280 nm, it was suggested that they include tryptophan residues; however, no amino acid was detected in peptide e after acid hydrolysis. Tryptophan was eluted between peptide a and b under the conditions. Therefore, peptide e was identified as Trp62–Trp63. On the basis of the above information, the identification of peptides a–e are also listed in Table 1. Thus, it was elucidated that the primary structure of the refolded lysozyme was identical to that of untreated native lysozyme.

On the other hand, the primary structure of the refolded OX62 lysozyme was determined according to the same procedures. The elution pattern of the tryptic peptides obtained from the refolded OX62 lysozyme is shown in Figure 2D. For comparison, the pattern of untreated OX62 lysozyme is shown in Figure 2C. There were no differences in the patterns between spectra C and D in Figure 2. The peptide corresponding to T_9+T_{11} in Figure 2C was further digested with α-chymotrypsin and prolylendopeptidase to examine the disulfide pairs among Cys64, Cys76, Cys80, and Cys94 (Figure 3B). There were similar patterns between spectra A and B in Figure 3 even though the peptides corresponding to d and e in Figure 3A were eluted earlier than those in Figure 3B because of the conversion of tryptophan 62 to oxindolealanine. Moreover, from amino acid analyses of peptides in Figure 3B, the formation of disulfide pairs among the four cystines was the same as that of untreated lysozyme (data not shown). Therefore, the primary structure of the refolded OX62 lysozyme was concluded to be identical to that of the untreated one. Thus, from the above results, the reason why the lytic activity of the refolded OX62 lysozyme decreased was strongly suggested to be due to the alteration of the higher-order structure in the refolded OX62 lysozyme.

Higher-Order Structures of the Refolded Lysozymes. As for the higher-order structure of the refolded native lysozyme, on the basis of the CD spectra the secondary structure was suggested to be the same as that of native untreated lysozyme. NMR is a good tool to detect conformational changes; therefore, to examine the tertiary structure of the refolded lysozyme, two-dimensional NMR analysis was carried out. Because the fingerprint region of the phase-sensitive ^1H–^1H COSY spectrum of the refolded sample was identical to that of untreated lysozyme, the tertiary structure of the refolded sample was found to be identical to that of native lysozyme. On the basis of the above results, we concluded that the structure of the refolded lysozyme (*ca.* 95% yield),

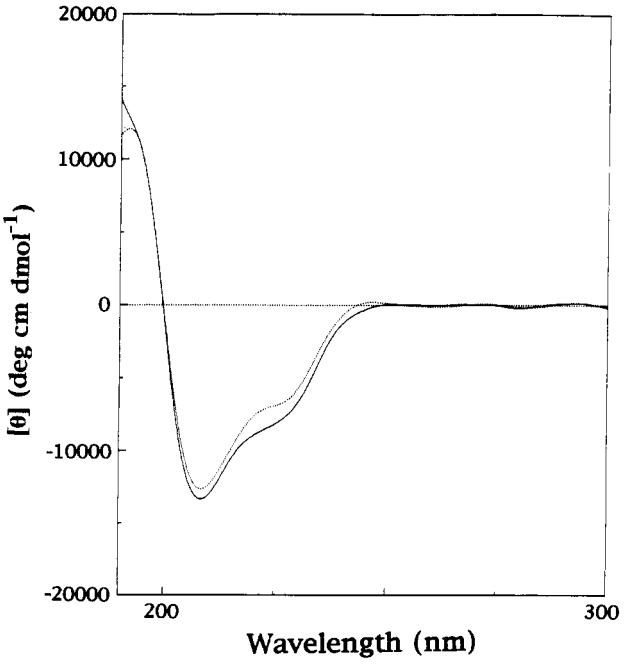

FIGURE 5: Circular dichroism spectra of untreated OX62 lysozyme (- - -) and the refolded OX62 lysozyme (—) at pH 7 and 20 °C.

which was separated by acid–urea gel chromatography, was identical to that of native lysozyme.

On the other hand, on the basis of the CD spectrum, the secondary structure of the refolded OX62 lysozyme was only slightly different from that of native lysozyme (Figure 5). The difference was so small that it may be neglected, as has been done with several lysozymes that mutated in the active site (Inoue *et al.*, 1992a,b). However, to investigate the tertiary structure of the refolded OX62 lysozyme further, its phase-sensitive ^{1}H–^{1}H COSY spectrum was measured. The fingerprint region of the derivative is shown in Figure 6B. For comparison, that of the untreated OX62 lysozyme is also shown in Figure 6A. The assignments of the proton resonances were carried out on the basis of ^{1}H–^{1}H phase-sensitive COSY and ^{1}H–^{1}H NOESY by reference to the assignments in the literature (Redfield & Dobson, 1988). Clearly, there were some differences in the pattern between the refolded OX62 lysozyme and the untreated one. The difference in the chemical shifts of the CαH and the NH resonances between the refolded OX62 lysozyme and the untreated one are plotted in Figure 7A,B as a function of residue number. There were some differences in the chemical shifts in the vicinity of the modification site. The modification site was located in the active site cleft; therefore, these results may be involved in the decrease in activity of the refolded OX62 lysozyme.

Oxindolealanine 62 is known to exist as an equimolar mixture of two diastereomers in untreated OX62 lysozyme (Norton & Allerhand, 1976; Blake *et al.*, 1981). In untreated OX62 lysozyme, the CαH–NH cross-peaks of the two diastereomers could not be detected by peak broadening because the residue had many conformations due to high exposure to the solvent (Imoto *et al.*, 1972). This result was consistent with that in a previous report (Blake *et al.*, 1981). However, the shifts in the CαH–NH cross-peak frequencies at the residues around the modification site, except for the position 62, could be observed (Figure 6A). These shifts in cross-peak frequencies may have been caused by two

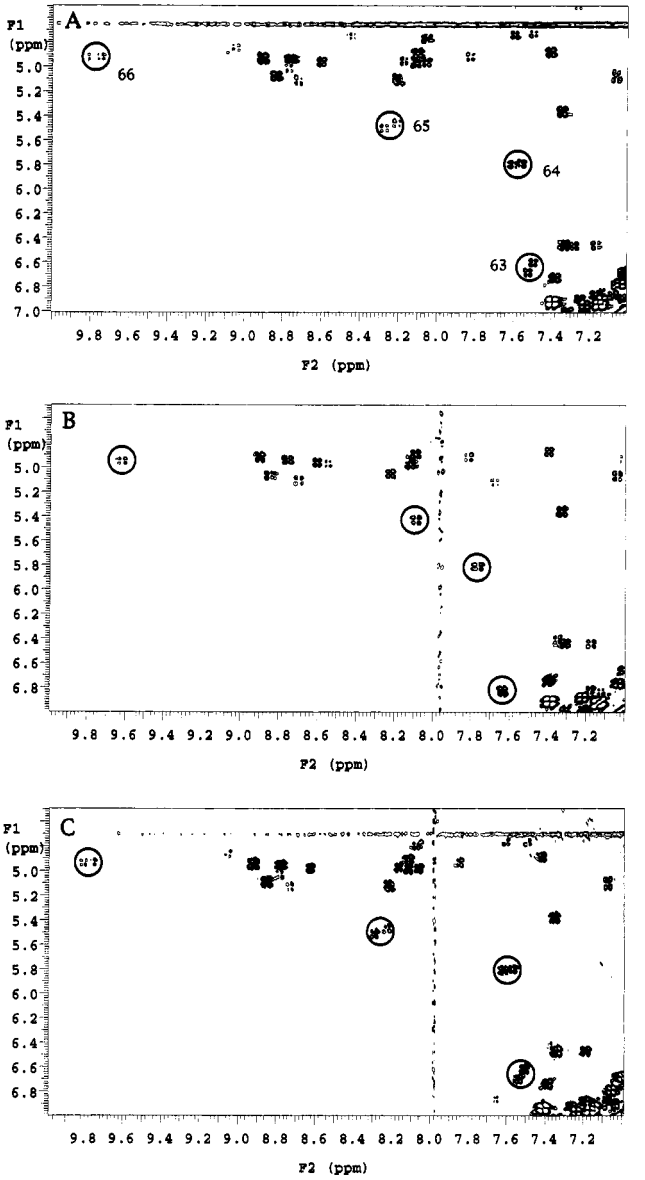

FIGURE 6: Contour plot of the fingerprint region (CαH–NH) of the phase-sensitive COSY spectra for the untreated OX62 lysozyme (A), the refolded OX62 lysozyme (B), and the refolded OX62 lysozyme after denaturation–renaturation treatment (C). Significant cross-peaks are surrounded with circles. The spectra were measured at a protein concentration of 2 mM at pH 3.8 and 35 °C.

diastereomers of oxindolealanine 62. On the other hand, in the fingerprint region of the two-dimensional spectrum of the refolded OX62 lysozyme, the shifts in CαH–NH cross-peak frequencies at the residues around the modification site could not be observed (Figure 6B). However, a cross-peak resulting from oxindolealanine 62 in the refolded OX62 lysozyme could not be assigned. As described above, because the Trp62 residue was fully exposed to the solvent (Imoto *et al.*, 1972), we may exclude the possibility that coalescence in CαH–NH cross-peaks at the residues around the position at 62 in the refolded OX62 lysozyme resulted from the fast exchange between the two diastereomers due to further exposure to oxindolealanine. Therefore, coalescence of the shifts in the CαH–NH cross-peak frequencies at the residue around position 62 in the refolded OX62 lysozyme strongly suggests that OX62 was trapped in one conformer of the diastereomers by the structural hindrance.

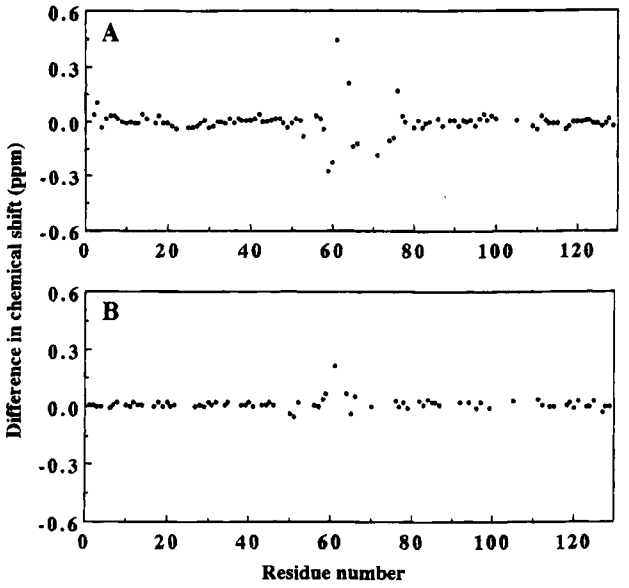

FIGURE 7: Plots of the chemical shift difference versus amino acid sequence for untreated OX62 lysozyme minus the refolded OX62 lysozyme for the CαH protons (A) and the NH protons (B). No difference value was plotted when the assignment for either of the two proteins was missing.

On the basis of affinity chromatography previously reported, after one conformer in two diastereomers is predominantly formed in the early stages of folding of the unfolded state of OX62 lysozyme under nonreduced conditions, it was gradually converted to the other toward equilibrium (Ueda *et al.*, 1990). Therefore, we examined whether the refolded OX62 lysozyme has a structure similar to one conformer of the diastereomers that appeared in the early stage of folding of the unfolded state of OX62 lysozyme under nonreduced conditions. Untreated OX62 lysozyme, 100 mg, was dissolved in 100 μL of 10% D_2O solution at pH 3.8 containing 6M Gdn-HCl and incubated at 40 °C for 1 h. Then, the unfolded OX62 lysozyme was rapidly diluted 50 times to a 10% D_2O solution at pH 3.8. ^{1}H-NMR spectra in the indole proton region after 20 min and 2.5 h of dilution, respectively, were measured at 35 °C (Figure 8B,C). These spectra were almost identical to each other, and they were similar to that of the untreated OX62 lysozyme (Figure 8A) but not to that of the refolded OX62 lysozyme. (Figure 8D). From these results, we concluded that one of the conformers that appeared in the early stages of folding from the unfolded state of OX62 lysozyme under nonreduced conditions had a structure different from the refolded OX62 lysozyme.

NOESY Analysis of the Refolded OX62 Lysozyme. In the untreated OX62 lysozyme, it was hard to detect the proton resonance of the side chains in oxindolealanine 62 and Trp63 because of their broadening as described in the literature (Blake *et al.*, 1981). However, both of the cross-peaks between the Trp63 C6H proton and Ile98 CαH proton and between the Trp63 C5H proton and Leu75 δCH3 proton were observed on the NOESY spectrum of the refolded OX62 lysozyme (Figure 9A,B). They were assigned by both COSY and NOESY spectrum. These NOEs could not be observed in the untreated OX62 lysozyme, whereas the distance between the Trp63 C6 and Ile98 Cα atoms and between the Trp63 C5 and Leu75 δC atoms were 3.9 and 4.1 Å, respectively, based on the X-ray crystallographic data of native lysozyme (PDB 1HEL). These results indicate that the movement of the side chain of Trp63 was restricted in the refolded OX62 lysozyme. In the present case, we could not detect the interaction of OX62 with the α-helix 88−98 but we could detect that of the Trp63 residue. The behavior in the Trp63 residue was shown to be reflected on that of OX62 in an earlier NMR analysis of OX62 lysozyme (Blake *et al.*, 1981). Moreover, the Trp63 residue was supposed to be affected by OX62 in the unfolded state because there were interactions between the amino acid side chains in the sequence of Trp-Trp or Trp-Tyr (Rizzo & Jackle, 1983). Therefore, Trp62 may be confirmed to be one of the key residues in the renaturation of reduced lysozyme. This idea was supported by the result that the interaction of the helix with Trp62 and/or Trp63 was present in the early stages of

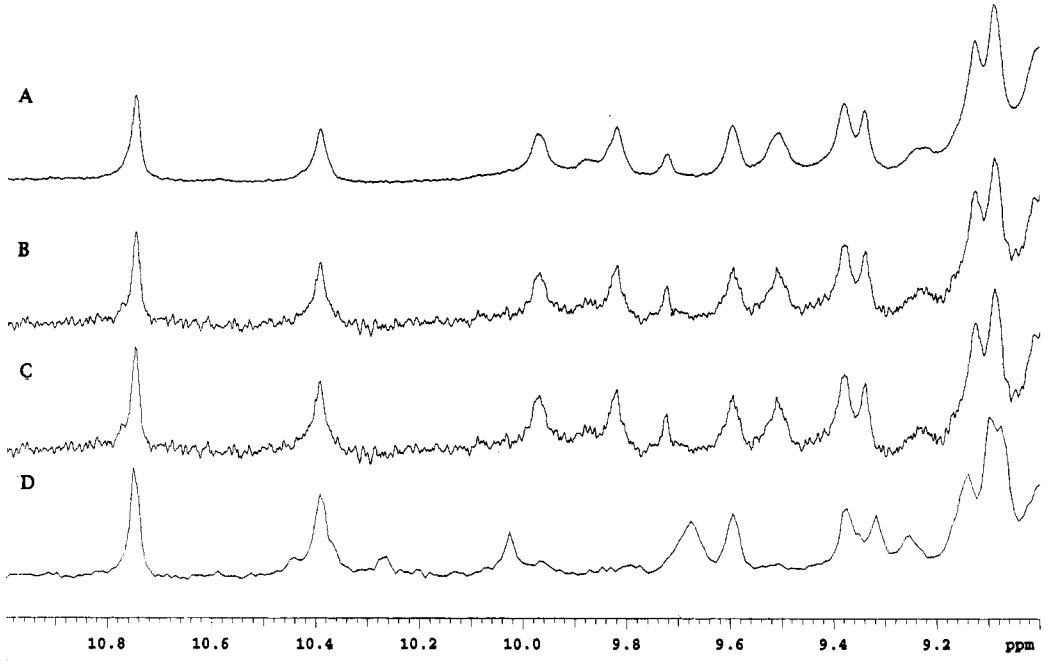

FIGURE 8: ^{1}H-NMR spectra of OX62 lysozyme at pH 3.8 and 35 °C. The untreated OX62 lysozyme (A); the sample after 20 min (B) or 2.5 h (C) of the dilution of unfolded OX62 lysozyme under nonreduced conditions; the refolded OX62 lysozyme (D).

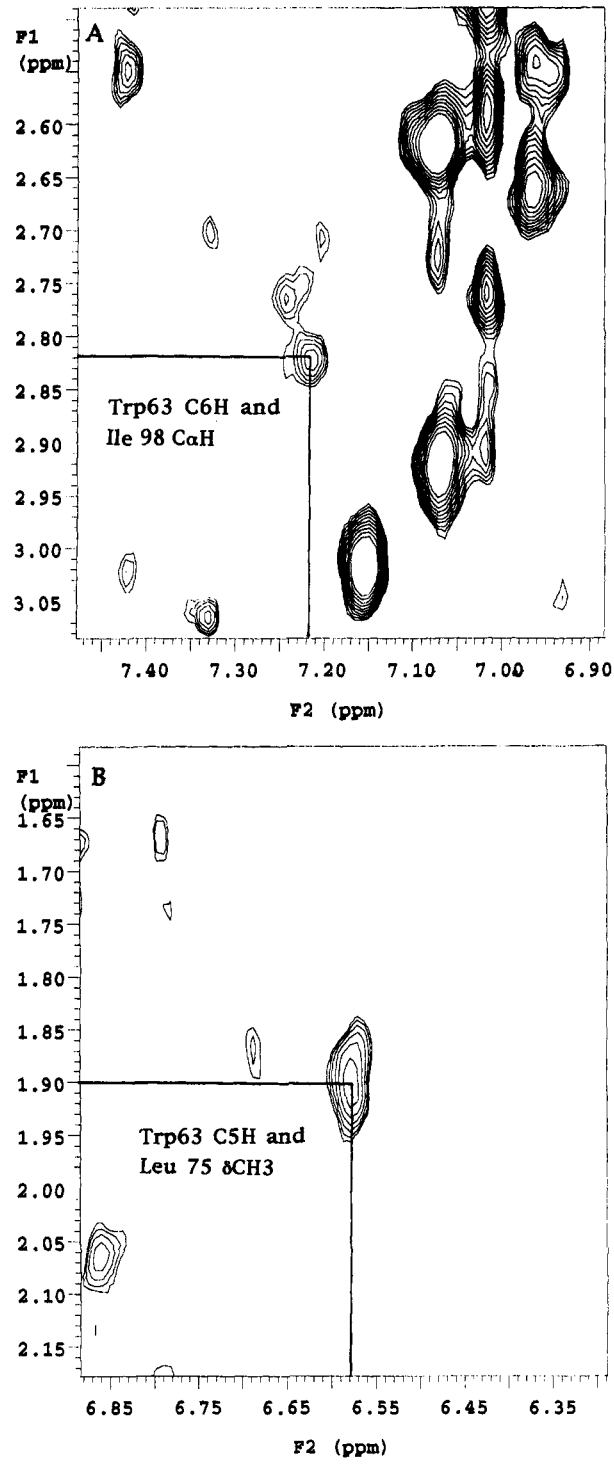

FIGURE 9: NOESY spectrum of the refolded OX62 lysozyme at pH 3.8 and 35 °C. (A), the cross-peak between Trp63 C6H and Ile98 CαH; (B), the cross-peak between Trp63 C5H and Leu75 δCH3 are assigned on the chart.

the folding of reduced lysozyme using a mutant lysozyme and peptide fragments (Ueda *et al.*, 1994).

Analysis of the Unstable Species in the Refolding Product of Reduced OX62 Lysozyme. After the reduced native lysozyme was refolded by the catalysis reaction with both 2-mercaptoethanol provided by the reduction mixture and oxidized glutathione, it was reacted with *N*-ethylmaleimide (see Materials and Methods). The reaction mixture was immediately applied to the column of the Sephadex G-75 (3 cm × 180 cm) (Figure 1B). Here we investigated the former peak in Figure 1B. When the derivative in this peak is treated with BrCN, digested by lysylendopeptidase, and analyzed on RP-HPLC, the peaks were very broad, whereas the peptides derived from untreated OX62 lysozyme produced three sharp peaks from this treatment. After incubation of the peptides derived from the former peak with 2-mercaptoethanol, the peaks became sharp but were different from those from untreated OX62 lysozyme due to the prior partial modification of cystines with *N*-ethylmaleimide. Moreover, one disulfide bond was found to be broken because one cysteine modified with *N*-ethylmaleimide per molecule could be detected in the former peak of the derivative by analyzing the amino acid composition. These results indicate that the former peak consisted of mixtures with incomplete formation of disulfide bonds. The arrow in Figure 2B indicates the elution position of BrCN-cleaved untreated OX62 lysozyme. This suggests that the introduction of the nick into the molecule and/or mislinkage of the disulfide bond caused the destabilization and it eluted earlier on gel chromatography. This result supports the idea that the former peak was a mixture of proteins with incomplete disulfide bonds. In human lysozyme, whose structure is homologous to hen lysozyme, rapid intraconversion among the four cystines (Cys65, Cys77, Cys81, and Cys95), which are closely located in space, took place in the mutant of Cys81 to Ala (Taniyama *et al.*, 1990). Therefore, it may be difficult to analyze the location of the cleaved disulfide bond. The modification of Trp62 residue might hinder the formation of cystine around the modification site and produce the derivative in the former peak.

Thermodynamically Stable Structure of the Refolded OX62 Lysozyme under Nonreduced Conditions. As described above, the refolded OX62 lysozyme had the same primary structure but a different tertiary structure relative to the untreated one. Because the primary structure of the refolded OX62 lysozyme was identical to that of the untreated one, we investigated whether the refolded OX62 lysozyme could have an untreated conformation. The refolded OX62 lysozyme (20 mg) was dissolved in 2 mL of 0.1 M Tris HCl buffer at pH 8 containing 6 M Gdn-HCl and incubated for 60 min at 40 °C. The protein was dialyzed against the same buffer without the denaturant, and then it was dialyzed against distilled water exhaustively and lyophilized. Two-dimensional NMR provides the sensitivity to detect conformational changes; therefore the $^1H-^1H$ COSY spectrum of the sample was measured (Figure 7C). The pattern closely resembled that of the untreated one, and double cross-peaks between CαH and NH appeared, which resulted from two diastereomers as observed in untreated OX62 lysozyme. This strongly suggests that the majority of the refolded OX62 lysozyme was changed to an untreated one by the denaturation–renaturation treatment under the nonreduced conditions.

On the other hand, the refolded OX62 lysozyme (20 mg) was dissolved in 2 mL of 0.1 M Tris-HCl buffer containing 10% D_2O at pH 8 without denaturant and incubated at 40 °C. The volume of the cross-peaks around residue 62 in the refolded OX62 lysozyme barely decreased (less than 5%) after incubation for 3 days. This result indicates that there is a high-energy barrier (above 28 kcal/mol) in the intraconversion between the refolded OX62 lysozyme and the untreated one without the denaturant, whereas the activation free energy change from the folded state to the unfolded state was estimated to be 23.5 kcal/mol by use of the unfolding

rate constant of native lysozyme (2.2×10^{-4} s^{-1}; Imoto *et al.*, 1986) at pH 8 and 40 °C. From these results, the refolded OX62 lysozyme was elucidated to be a metastable and kinetically trapped product from the renaturation process of reduced lysozyme. On the other hand, as previously described (Ueda *et al.*, 1990), the unfolded OX62 lysozyme was finally converted to untreated OX62 lysozyme by renaturation under nonreduced conditions. Therefore, the formation of the refolded OX62 lysozyme during the renaturation of reduced OX62 lysozyme may depend more on favorable interactions between the region around OX62 and α-helix 88−98 because of the release of the restriction by disulfide bonds.

Wetlaufer *et al.* (1974) demonstrated that reduced lysozyme could be refolded at higher temperatures where native lysozyme is unstable. However, in this case, active lysozyme was formed as a transient intermediate. In the present case, it would be novel that the refolded OX62 lysozyme is shown to be a metastable and kinetically trapped product from the renaturation process. While the modification of the Trp residue to oxindolealanine may not occur in nature, the modification itself is very minor. Therefore, it may be possible that the modification, including the mutation of an amino acid, gives the final product a drastic alteration in its conformation. We should pay more attention to the conformation of the final folded product.

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