Serine proteinase inhibitors (serpins) are believed to fold in vivo into a metastable “stressed” state with cleavage of their P1-P1′ bond resulting in reactive center loop insertion and a thermostable “relaxed” state. To understand this unique folding mechanism, we investigated the refolding processes of the P1-P1′-cleaved forms of wild type ovalbumin (cl-OVA) and the R339T mutant (cl-R339T). In the native conditions, cl-OVA is trapped as the stressed conformer, whereas cl-R339T attains the relaxed structure. Under urea denaturing conditions, these cleaved proteins completely dissociated into the heavy (Gly1–Ala352) and light (Ser353–Pro385) chains. Upon refolding, the heavy chains of both proteins formed essentially the same initial burst refolding intermediates and then reassociated with the light chain counterparts. The reassociated intermediates both refolded into the native states with indistinguishable kinetics. The two refolded proteins, however, had a notable difference in thermostability. cl-OVA refolded into the stressed form with $T_m = 68.4 \degree C$, whereas cl-R339T refolded into the relaxed form with $T_m = 85.5 \degree C$. To determine whether cl-R339T refolds directly to the relaxed state or through the stressed state, conformational analyses by anion-exchange chromatography and fluorescence measurements were executed. The results showed that cl-R339T refolds first to the stressed conformation and then undergoes the loop insertion. This is the first demonstration that the P1-P1′-cleaved serpin peptide capable of loop insertion refolds to the stressed conformation. This highlights that the stressed conformation of serpins is an inevitable intermediate state on the folding pathway to the relaxed structure.

The serpins are believed to fold in vivo into a metastable “stressed form” as they undergo a conformational change to the thermostable “relaxed” form by insertion of the reactive center loop (RCL) between strands 3A and 5A (see Fig. 1) (1–3). The metastability of the native serpin is essential for the inhibition of serine proteinases, although this molecular flexibility also results in aberrant intermolecular linkage and polymerization that bring about conformational diseases such as dementia (4, 5), hepatocirrhosis (6), angioedema (7), and emphysema (8, 9). Therefore a central target of serpin studies is how the serpin polypeptide can fold into a metastable structure avoiding thermodynamically more stable conformations. Several serpin folding models have been suggested by various unfolding experiments, whereas refolding experiments from a fully unfolded state are limited (10–14) because of serpin susceptibility to misfolding and aggregation.

Ovalbumin is a major component of egg white proteins, and although it is not an apparent protease inhibitor its membership of the serpin family of protease inhibitors is clear from the close similarity of its primary and tertiary structure (15, 16). We have elucidated its refolding process from a fully denatured state as (10, 17, 18)

$$D_{N-SS} \rightarrow I_{N-SS} \rightarrow N_{N-SS}$$

\[ \text{SCHEME 1} \]

where $D$, $N$, and $I$ represent urea-denatured ovalbumin, native ovalbumin, and an initial burst refolding intermediate with the native disulfide Cys$^\text{73}$-Cys$^\text{120}$ (subscript N-SS) or a mispaired disulfide (subscript mis-SS), respectively. Ovalbumin contains a disulfide (Cys$^\text{73}$-Cys$^\text{120}$) and four sulfhydryls (Cys$^\text{11}$, Cys$^\text{30}$, Cys$^\text{367}$, and Cys$^\text{382}$), and disulfide rearrangements (dashed arrows) occur in non-native states at neutral pH conditions. $D_{N-SS}$ can be prepared by denaturation of ovalbumin in acidic urea conditions ($9 \text{M urea, pH 2.0}$) where disulfide rearrangements are blocked. $I_{N-SS}$ is formed by dilution of $D_{N-SS}$ with a neutral pH buffer within a time scale of milliseconds. This intermediate folds to $N_{N-SS}$ and concurrently transforms to a cleaved ovalbumin; CM-OVA, uncleaved ovalbumin derivative with a mispaired disulfide Cys$^\text{73}$-Cys$^\text{120}$ and carboxymethylated Cys$^\text{352}$; RCL, reactive center loop; DTT, dithiothreitol; CD, circular dichroism; $T_m$, melting temperature; ANS, anilino-1-naphthalene-8-sulfonate; FRET, fluorescence resonance energy transfer; HPLC, high pressure liquid chromatography; int-R339T, intact R339T; cl-R339T, P1-P1′-cleaved R339T; Tricine, N-[2-hydroxy-1, 1-bis(hydroxymethyl)ethyl]glycine; H-R339T, heavy chain (Gly$^\text{1}$–Ala$^\text{352}$) of P1-P1′-cleaved R339T.
In this study, we analyzed the refolding processes of cl-OVA and cl-R339T from a fully denatured state where these cleaved proteins are completely separated into their heavy (Gly1–Ala352) and light (Ser353–Pro385) chains. We demonstrated that cl-OVA and cl-R339T refold to the stressed and the relaxed forms, respectively, after reassociation of the heavy and light chains in a refolding intermediate state. Furthermore we found that the relaxed structure of refolded cl-R339T is formed via the stressed conformation during refolding. This is the first report that the P1-P1’-cleaved serpin peptide capable of loop insertion refolds to the stressed conformation. On the basis of the observations, we propose a model for the refolding pathways of cleaved serpins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Intact wild type ovalbumin (int-OVA) from egg white (29, 30) and intact R339T (int-R339T) (31, 32) were purified as described previously. cl-OVA and cl-R339T were prepared by incubation of a 225 μM concentration of the intact forms in 0.1 M sodium phosphate buffer, pH 7.0, at 25 °C for 2 h with 60 μg/ml porcine pancreatic elastase Type I (EC 3.4.21.36, purchased from Sigma). The limited proteolysis was stopped by the addition of 0.015 volume of 0.1 M phenylmethylsulfonyl fluoride 2-propanol solution. After the buffer was replaced by 50 mM sodium phosphate buffer, pH 6.0, using a Sephacryl G-10 column (GE Healthcare NAP-10) at 2 °C, the obtained sample was loaded onto a UNO Q-6 column (Bio-Rad) equilibrated with the buffer, and then the bound protein was eluted with a sodium phosphate gradient (50–150 mM). The fractions of the cleaved form were collected and checked by Tricine SDS-PAGE (33). The protein concentrations of the samples were estimated from the absorption at 280 nm by the method of Pace et al. (34).

For preparation of the disulfide-reduced proteins, 0.3 mM disulfide-bonded proteins were incubated with 15 mM DTT at 37 °C for 2 h in 50 mM Tris-HCl, 1 mM Na-EDTA, pH 8.2.

**Preparation of the Heavy and Light Chains of Cleaved Ovalbumin**—The urea-denatured heavy (Gly1–Ala352) and light (Ser353–Pro385) chains were separated into two distinct peaks as detected by absorbance at 220 nm. The early and late fractions were found by Tricine SDS-PAGE (33) to correspond to the heavy chain (H-OVA or H-R339T) and the light chain, respectively. The N-terminal sequence of the late peptide fraction was analyzed with a sequenator, Procise 492 (Applied Biosystems), and determined to be Ser-Val-Ser, which corresponded to the P1-P1’-P3’ residues (Ser353-Val354-Ser355) of ovalbumin. For further analyses, the separated heavy and light chains were concentrated with a concentrator (Millipore Centriprep-10 and -3), and the protein concentration was adjusted using acid/urea solution. The molar concentration of the heavy chain was esti-
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mated from the absorption at 280 nm by the method of Pace et al. (34), and that of the light chain was estimated on the basis of the molar concentration of the cysteine residues (Cys^{367} and Cys^{382}) determined by use of 5,5'-dithiobis-2-nitrobenzenzoic acid (35). The disulfide-bonded H-OVA for the disulfide rearrangement experiment was prepared from the non-reduced cl-OVA in the same manner.

Refolding Experiments—Refolding was initiated by 20-fold dilution of 22.5 μm urea-denatured protein with refolding buffer (50 mM Tris-HCl, 0.5 mM DTT, 1 mM Na-EDTA, pH 8.6) giving a final pH of 8.2. The protein was allowed to refold at 25 °C. The partially refolded heavy chain I_{ν}-H was prepared by 19.5-fold dilution of the urea-denatured heavy chain with refolding buffer, and the further refolding to the native state was initiated by the addition of 1/6 volume of the urea-denatured light chain to the I_{ν}-H sample. CM-OVA, which is an uncleaved ovalbumin derivative with a mispaired disulfide Cys^{367}-Cys^{382} and carboxymethylated Cys^{73}, was prepared as described previously (10). I_{ν}-CM-OVA, which is a partially refolded ovalbumin staying in the initial burst refolding intermediate state (10), was prepared by 20-fold dilution of urea-denatured CM-OVA with non-DTT-containing refolding buffer. The buffers were degassed at reduced pressure and equilibrated under N_{2} atmosphere prior to the refolding.

Intrinsic Trp Fluorescence Measurement—The intrinsic Trp residues in the protein were excited at 295 nm, and the emission spectrum was recorded with a fluorescence spectrophotometer (Jasco FP-720). For the spectrum measurements at a short refolding time, the time course of fluorescence intensity change was monitored at various emission wavelengths, and the data at a refolding time of 10 s were plotted. The time course of the refolding after the initial burst phase was monitored by the fluorescence intensity change at 338 nm. The fraction of the native form at the refolding time of t, "F_{ν}(t)," were calculated by the equation \( F_{ν}(t) = (X_{ν} - X_{ν})/(X_{ν} - X_{ν}) \) where \( X_{ν} \) and \( X_{ν} \) are the fluorescence intensity of the native form, the value of the 6-s refolding, and the value at the refolding time t, respectively. All Trp fluorescence measurements were carried out at a constant temperature of 25 °C, and the data were obtained from an average of three to six determinations. The kinetic analysis was performed using the Levenberg-Marquardt algorithm (36).

The time course of very early refolding was measured by intrinsic Trp fluorescence (excitation, 295 nm; emission, 338 nm) with a stopped-flow reaction analyzer (Applied Photophysics Ltd.). The urea-denatured heavy chain was mixed with a 9-fold volume of refolding buffer, and then the fluorescence change was recorded. For the control, mixing experiments of the urea-denatured heavy chain with acid/urea solution and of the non-denatured cleaved protein with refolding buffer containing 0.8 mM urea were carried out. The data were obtained from the average of 10–15 traces. The dead time for mixing was determined to be 4 ms by a model reaction between 2,6-dichlorophenolindophenol and L-ascorbate (37).

Far-UV CD Measurement—The far-UV CD spectrum was recorded at 25 °C with a spectropolarimeter (Jasco J-720). The path length and slit width were 0.01 cm and 1 nm, respectively. The averages of 10 traces were determined. The samples, except for the urea-denatured proteins, were concentrated and dialyzed with 20 mM sodium phosphate buffer, pH 7.8, using a concentrator (Millipore Amicon Ultra-4).

ANS Binding Experiments—Anilino-1-naphthaleine-8-sulfonate (ANS) was excited at 350 nm, and the emission spectra were recorded at 25 °C with a fluorescence spectrophotometer (Jasco FP-720). The protein samples (1.13 μm) in the various states were mixed with 0.005 volume of 4.02 mm ANS, and the measurements were started after 20 s of mixing.

Size Exclusion Chromatography—The refolded samples were obtained by incubation of I_{ν}-H in the presence of an equimolar amount of the light chain at 25 °C for 24 h and then concentrated with a concentrator (Millipore Amicon Ultra-4). The obtained samples and non-denatured protein controls were analyzed at 25 °C and a flow rate of 0.5 ml/min with a TSK gel column (Tosoh G3000SWXL) equilibrated with 20 mM Tris-HCl buffer, pH 8.2, containing 1 mM 2-mercaptethanol. Urea-denatured H-OVA, H-R339T, and the light chain were also subjected to this analysis using the same column equilibrated with the acid/urea solution.

Trypsin Resistance Assay—The trypsin resistance assay was carried out as described elsewhere (10, 17). Briefly the sample was mixed with 0.01 volume of 12.5 mg/ml trypsin and digested at 25 °C for 1 min. The digestion was terminated by the addition of soybean trypsin inhibitor. The proteins were separated by SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250.

Analysis for Disulfide-involved Cysteines—Urea-denatured disulfide-bonded H-OVA in acid/urea solution was refolded by dilution with non-DTT-containing refolding buffer, and the further refolding was initiated by the addition of an equimolar amount of the light chain. Sulphhydryl/disulfide exchanges were quenched at various refolding times by mixing the protein samples with 0.24 volume of 2 mM HCl. Disulfide-involved cysteines were determined by a peptide mapping analysis with reverse phase HPLC as described elsewhere (10, 17). The data were obtained from the average of quadruplicate determinations.

Differential Scanning Calorimetry—The refolded samples were prepared in the same manner as described under “Size Exclusion Chromatography” and dialyzed with 10 mM sodium phosphate buffer, pH 6.0, using a concentrator (Millipore Amicon Ultra-4). The refolded proteins and the non-denatured protein controls at 11 μm were analyzed with a differential scanning calorimeter (Micro Cal MCS-DSC) at the heating rate of 1 °C/min⁻¹.

Analysis for the Stressed and Relaxed Conformations by Anion-exchange HPLC—The mixture of I_{ν}-H and the light chain was allowed to refold at 25 °C in the same conditions as described above, and then an aliquot at various refolding times was diluted 5-fold with 0.15 M Tris-HCl, 20 mM NaCl buffer, pH 8.0, prechilled at 0.5 °C. The sample was kept at 0.5 °C and applied to Mono Q 5/50 GL or 10/100 GL (GE Healthcare) equilibrated with 0.15 M Tris-HCl, 20 mM NaCl buffer, pH 8.0, at 0.5 °C. The bound protein was eluted with an NaCl gradient (20–150 mM) and then with 1 mM Tris base, 1 mM NaCl, pH 10.6. The peaks were analyzed using a Bowin system (Jasco), and the kinetic curve-fitting analysis was performed using the Levenberg-Marquardt algorithm (36). The data were obtained from the average of quadruplicate determinations.
Fluorescence Resonance Energy Transfer (FRET) Analysis of the Stressed and Relaxed Forms—The proteins obtained by the HPLC analysis described in the preceding section were concentrated with a concentrator (Millipore Ultrafree-0.5) at 0.5 °C. The protein concentrations of the obtained samples and the non-denatured protein controls were determined by protein assay (Bio-Rad) and adjusted to 20 μM with prechilled Tris buffer (50 mM Tris-HCl, pH 8.0). The samples were maintained at 0.5 °C until mixing with ANS or subjected to incubation at 25 °C for 1 h followed by cooling at 0.5 °C for 5 min. The chilled sample was mixed with 19 volumes of 0.105 mM ANS in Tris buffer at 4 °C and then incubated for 20 min at the same temperature. It was confirmed by the CD analysis described in the supplemental data that the protein refolding was quenched at 4 °C at least for 4 h. The Trp residues of the protein were exited at 295 nm, and ANS fluorescence was scanned from 410 to 580 nm at 4 °C within 1 min by use of a fluorescence spectrophotometer (Jasco FP-720) with a temperature control and N₂ stream system (Jasco ETC-272). The data were obtained from the average of three determinations.

RESULTS

Refolding of Cleaved Ovalbumin Detected by Spectroscopic Analyses—Ovalbumin has disulfide bond Cys⁷³-Cys¹²⁰, but its conformational structure is indistinguishable from that of the disulfide-reduced form (38, 39). In addition, fully denatured ovalbumin can refold to the native structure without the formation of a disulfide linkage (17, 38, 39). Thus refolding experiments in this study, except for the refolding of CM-OVA and the disulfide rearrangement analysis (see Fig. 4), were principally carried out under the disulfide-reduced conditions, which give advantages in the refolding yield and in preventing a cross-linkage between the heavy and light chains by a disulfide bond.

The conformational change of the heavy chain during refolding was analyzed by intrinsic Trp fluorescence and far-UV CD (Fig. 2, a and b). In the denaturing conditions, H-OVA and H-R339T showed the typical fluorescence and CD spectra of the unfolded protein. After initiating the refolding by dilution with refolding buffer, both H-OVA and H-R339T immediately formed a partially refolded intermediate, IN-H, that had the same maximum fluorescence at 338 nm as native int-OVA and 56% of CD intensity at 222 nm as compared with native int-OVA. IN-H was formed within an instrumental dead time of 4 ms (Fig. 2a, inset), and the fluorescence spectra at 10 s of refolding were almost indistinguishable from those at 2 h of refolding (data not shown). These characteristics are consistent with those of IN-CM-OVA, which is an uncleaved ovalbumin derivative staying in the initial burst refolding intermediate state (10).

ANS is a widely used probe for detecting an exposed hydrophobic core in proteins as it exhibits a marked increase in emission upon binding a hydrophobic cluster (40). Our previous

FIGURE 2. Refolding of cleaved ovalbumin monitored by intrinsic Trp fluorescence (a), far-UV CD (b), and ANS fluorescence (c). Solid curves with N, int-OVA (green), cl-OVA (black), and cl-R339T (red) in the native conditions. Thin solid curves with D, int-OVA (green), H-OVA (black), and H-R339T (red) in the urea denaturing conditions. Dotted curves with IN, IN-CM-OVA (green), IN-H-OVA (black), and IN-H-R339T (red) were prepared by incubation for 2 h at 25 °C after dilution of the urea-denatured H-OVA and H-R339T, respectively. IN-CM-OVA is a partially refolded uncleaved ovalbumin staying in the initial burst refolding intermediate state (10). Dashed curves with R, the further refolded protein obtained by incubation of IN H-OVA (black) or IN H-R339T (red) in the presence of an equimolar amount of the light chain for 2 h at 25 °C. The arrow with L, the light chain in the refolding conditions. In the inset of a, urea-denatured H-OVA was refolded, and the changes in fluorescence at 338 nm were recorded using a stopped-flow tool. For comparison, cl-OVA in the native conditions (N) and H-OVA in the urea denaturing conditions (D) were also recorded. This stopped-flow experiment was also carried out with R339T, and almost the same result was obtained (data not shown). deg, degrees.
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![Graph](image)

**FIGURE 3. Characterization of refolded cleaved ovalbumin.** a, elution profiles from size exclusion chromatography of the refolded cleaved proteins. The refolded sample was obtained by incubation of IN-H-OVA or IN-H-R339T with the light chain for 24 h at 25 °C. For control, non-denatured cl-OVA and cl-R339T were also analyzed. The respective elution volumes were 9.57 ml for cl-OVA and refolded cl-OVA and 9.81 ml for cl-R339T and refolded cl-R339T. The light chain was confirmed to be eluted, on the same column but under the denaturing conditions, with an elution volume (10.9 ml) different from those of H-OVA (6.55 ml) and H-R339T (6.69 ml). b, the samples collected in peak I (lane 3) and peak II (lane 6) were assessed by Tricine SDS-PAGE. For comparison, non-denatured proteins int-OVA (lane 1), cl-OVA (lane 2), int-R339T (lane 4), and cl-R339T (lane 5) were also loaded onto the gels. W, H, and L represent the whole peptide, the heavy chain, and the light chain, respectively. c, trypsin resistance of int-OVA (lanes 1 and 2), cl-OVA (lanes 3 and 4), the refolded protein from IN-H-OVA in the presence (lanes 5 and 6) or absence (lanes 7 and 8) of the light chain, int-R339T (lanes 9 and 10), cl-R339T (lanes 11 and 12), and the refolded protein from IN-H-R339T in the presence (lanes 13 and 14) or absence (lanes 15 and 16) of the light chain were assessed by SDS-PAGE. The samples in the lanes with even numbers were not treated with trypsin, and the bands within T are trypsin.

study has demonstrated that the initial burst refolding intermediate of int-OVA has a hydrophobic folding nucleus whose ANS binding ability is much higher than that of the native and urea-denatured proteins (10). Fig. 2c demonstrates that IN-H-OVA and IN-H-R339T have a hydrophobic core like the initial burst refolding intermediate of int-OVA.

When the light chain was added to IN-H-OVA and IN-H-R339T, these protein mixtures underwent further refolding into the native state as reflected by 94 and 89% recoveries of the fluorescence intensity of the respective native forms. These fluorescence increases could be accounted for by regain of the native structure because the Trp residues (Trp148, Trp184, and Trp367) of ovalbumin are all contained in the heavy chain (Fig. 1). The CD data endorse this regain of the native secondary structure in the further refolded proteins (Fig. 2b). When the light chain was added to the IN-H samples that had been precubated for a prolonged time of 30 min to 2 h, essentially the same native refolding was found (data not shown). These results strongly suggest that the heavy chain alone stays in the refolding intermediate IN-H in the refolding conditions but is capable of refolding into a native-like state after association with the light chain without the assistance of a disulfide bridge.

**Characterization of Refolded Cleaved Ovalbumin**—The preceding spectroscopic data suggest that the fully refolded protein comprises both the heavy and light chains. To confirm this, the refolded samples and the native protein controls were analyzed by size exclusion chromatography (Fig. 3a). The refolded proteins were eluted as a single peak at the same elution volume as the cleaved control proteins, and the peak fractions contained both the heavy and light chains (Fig. 3b). These data confirm that the heavy and light chains refold as a monomeric protein in the associated form.

The integrity of native refolding was assessed by a trypsin resistance assay because ovalbumin is highly resistant to trypsin in the native state but not in its non-native or its IN state (10, 38, 39). As shown in Fig. 3c, the refolded proteins from the mixture of IN-H and the light chain showed an almost complete resistance to trypsin as seen with the intact and cleaved forms in the native conditions. In contrast, the IN-N-H proteins did not show any resistance. These results are consistent with the conclusion that H-OVA and H-R339T can correctly refold into the native forms after reassociation with their light chain counterparts.

**Analysis of the Contacts between the Heavy and Light Chains during Refolding**—The native disulfide bond of ovalbumin, Cys73-Cys340, can be changed to a mispaired disulfide bond in the IN state by intrachain sulfhydryl/disulfide exchange reactions as shown in Scheme 1. To obtain direct evidence for the contact between the heavy and light chains, we examined interchain disulfide exchanges, utilizing wild type ovalbumin in which refolding analysis is possible under disulfide-bonded conditions. As shown in Fig. 4a, in the IN-H state, disulfide rearrangement reactions were found. Cys73 and Cys120 were detected as only the disulfide-involved cysteines in disulfide-bonded H-OVA in acid/urea solution (Fig. 4a, refolding time 0). After dilution of denatured H-OVA with non-DTT-containing refolding buffer, disulfide linkages involving Cys73 and Cys120 rapidly decreased and coincidentally Cys11 and Cys30 increased. The ratios of disulfide-involved cysteine at refolding time 120 min were 0.68 for Cys11, 0.77 for Cys30, 0.23 for Cys73, and 0.32 for Cys120, respectively. The greater ratios of Cys11 and Cys30 may largely result from their direct disulfide bonding as Cys11-Cys30.

The light chain has two sulphhydryls of Cys367 and Cys382 (Fig. 1). To analyze the contact between the heavy and light chains, the light chain was added to the IN-N-H-OVA sample at refolding time 60 min at which the ratio of disulfide-involved cysteine appears to reach equilibrium. As shown in Fig. 4b, disulfide-involved Cys367 rapidly increased after the mixing; the ratio was 0.24 at refolding time 0.5 min. This demonstrates that the light chain comes into immediate contact with IN-N-H-OVA. Strikingly, Cys382 showed very little participation in disulfide formation especially within refolding time 5 min. This implies that the light chain makes contact with IN-N-H-OVA with a specific coordination.

The increase of disulfide-involved Cys367 appears to be counterbalanced by the decrease of Cys30 within the refolding time 5 min (Fig. 4b, inset). This suggests that the sulphhydryl of Cys367...
initially attacks a half-cystine residue, $\frac{1}{2}\text{Cys}^X$, that forms a disulfide-linkage with Cys$^{30}$ and then forms a new disulfide linkage, Cys$^{367}$-$\frac{1}{2}\text{Cys}^X$, by the sulfhydryl/disulfide exchange reaction. Based on the result of Fig. 4a, $\frac{1}{2}\text{Cys}^X$ would be largely Cys$^{11}$. This is supported by the data that disulfide-involved Cys$^{11}$ decreased more slowly than Cys$^{30}$. These results are consistent with the view that the light chain interacts with $\text{I}_{\text{N}}$-$\text{H}$-OVA at a site where Cys$^{367}$ is readily accessible to Cys$^{11}$.

With the progress of refolding, the disulfide linkage involving Cys$^{73}$ and Cys$^{120}$ gradually increased. This indicates that $\text{I}_{\text{N}}$-$\text{H}$-OVA correctly refolds to cl-OVA with the native disulfide after reassociation with the light chain.

**Kinetics of Refolding after the Initial Burst Phase**—The refolding from the urea-denatured state to the $\text{I}_{\text{N}}$-$\text{H}$ state is completed within an instrumental dead time of 4 ms (Fig. 2a, inset), whereas the refolding after the initial burst phase is slow enough to monitor with a conventional photometer. The time course of the refolding after the initial burst phase was therefore analyzed by Trp fluorescence (Fig. 5). The progress curves of the cleaved proteins were very similar to that of the intact proteins when the refolding was started either from the mixture of the urea-denatured heavy and light chains or from the mixture of $\text{I}_{\text{N}}$-$\text{H}$ and the light chain (Fig. 5a). The data appeared to fit well to the monophasic curve, which is theoretically obtained from the data of the intact proteins (17). These data suggest that the cleaved forms refold through a pathway analogous to that of the intact forms.

Nevertheless the reassociation step of the heavy and light chains should be included in the refolding of the cleaved forms. The refolding pathway can be described as

$$\text{I}_{\text{N}}$-$\text{H} + \text{L} \rightarrow \text{I}_{\text{N}}$(H/L) $\rightarrow$ N(H/L)

where L, N(H/L), and $\text{I}_{\text{N}}$(H/L) represent the light chain, the native cleaved form, and the reassociated protein in the intermediate state, respectively. The constant $k_a$ is the second-order rate constant for the reassociation of the two chains, and $k_f$ is the first-order rate constant for the folding from $\text{I}_{\text{N}}$(H/L) to N(H/L). To estimate the rate constants, the $\text{I}_{\text{N}}$-$\text{H}$ samples (1.1 mM) were mixed with a 10-fold concentration of the light chain (11 mM), and then the refolding reactions were monitored by Trp fluorescence. Use of an excess amount of the light chain over the heavy chain enabled the reaction to be treated as a pseudo first-order reaction. As shown in Fig. 5b, the protein mixtures refolded with a slightly decelerated rate as compared with the intact forms. In this case, the reaction in Scheme 2 can be approximated as a two-step sequential first-order reaction, and the fraction of the native form at a refolding time $t$, $F_N(t)$, can be expressed as

$$F_N(t) = F_0 \times \left\{ 1 - k_f \exp\left(-k_f t\right)/\left(k_f - k_a\right) \right\} + \frac{k_a \exp\left(-k_a t\right)}{k_f - k_a} \quad (\text{Eq. 1})$$

where $F_0$ is the relative fluorescence intensity at $t = 240$ min at which the refolding is almost completed. $k_f$ is the pseudo first-order rate constant for the reaction from $\text{I}_{\text{N}}$-$\text{H}$ to N(H/L). On the other hand, the kinetics of int-OVA and int-R339T are given by the following equation as described previously (17).

$$F_N(t) = F_0 \times \left\{ 1 - \exp\left(-k_f t\right) \right\} \quad (\text{Eq. 2})$$

Fig. 5b shows that the time course of refolding in the presence of a 10-fold excess of the light chain fitted well to Equation 1. The obtained kinetic parameters shown in Table 1 demonstrate that the $\text{I}_{\text{N}}$(H/L) to N(H/L) step is rate-limiting for the refolding of the cleaved proteins. The large $k_f$ values are consistent with the rapid reassociation of $\text{I}_{\text{N}}$-$\text{H}$ and the...
light chain (Fig. 4b). The $k_f$ values for the cleaved forms were almost the same as those for the intact forms, indicating that the folding of $I_N(H/L)$ to $N(H/L)$ is substantially the same as that of intact $I_N$ to the native protein on monitoring by Trp fluorescence.

Thermostability of Refolded cl-OVA and cl-R339T—Our previous crystallographic and calorimetric studies have demonstrated that cl-R339T has a $T_m$ much higher than that of int-R339T because it forms a relaxed serpin structure (27). In contrast, cl-OVA shows a $T_m$ slightly lower than that of int-OVA because it keeps a stressed serpin form slightly destabilized by the P1-P1’ cleavage (41). To examine the

![FIGURE 5. Kinetics of refolding after the initial burst phase.](image)

![FIGURE 6. Thermostability of refolded cl-OVA and cl-R339T.](image)

| Protein   | $k_f$ (min$^{-1}$) | $k_a$ (min$^{-1}$) | $k_a$ (M$^{-1}$ min$^{-1}$) |
|-----------|------------------|------------------|---------------------------|
| int-OVA   | 0.25 ± 0.015     | 67 ± 0.011       | 6.1 × 10$^6$              |
| cl-OVA    | 0.24 ± 0.011     | 67 ± 0.011       | 6.1 × 10$^6$              |
| int-R339T | 0.23 ± 0.036     | 75 ± 2.5         | 6.8 × 10$^6$              |
| cl-R339T  | 0.22 ± 0.047     | 75 ± 2.5         | 6.8 × 10$^6$              |
thermostabilities of the refolded cleaved proteins, differential scanning calorimetry was carried out. As shown in Fig. 6, the $T_m$ of refolded cl-OVA (68.4 °C) was almost the same as that of non-denatured cl-OVA (68.3 °C) and slightly lower than that of intact OVA (70.7 °C). Also the $T_m$ of refolded cl-R339T (85.5 °C) was very similar to that of non-denatured cl-R339T (85.3 °C) and much higher than that of intact R339T (65.2 °C). These results demonstrate that cl-OVA and cl-R339T refold into the stressed and relaxed forms, respectively.

The Stressed to Relaxed Transition during Refolding—We have demonstrated previously that the RCL insertion of the P1-P1′-cleaved ovalbumin is almost completely quenched at pH 8 and 0.5 °C and that the pre-loop-inserted stressed form of P1-P1′-cleaved ovalbumin is eluted slightly later than the loop-inserted relaxed form in anion-exchange HPLC (42). To examine when the RCL insertion occurs during the refolding of cl-R339T, the samples at various refolding times were analyzed by the HPLC system (Fig. 7a). The refolding of cleaved ovalbumin was able to be quenched at 0.5 °C (see supplemental Fig. S1). In$_N$-H and the proteins at refolding time 0 min were not eluted within this gradient range (20–150 mM NaCl), but these non-native proteins were eluted in 1 M Tris base, 1 M NaCl, pH 10.6 (data not shown).

In the refolding of cl-OVA, a single peak Z increased in a monophasic manner during the progress of refolding, and its retention time was the same as that of non-denatured cl-OVA (6.67 min). In contrast, during the refolding of cl-R339T, two refolding products were found at retention times of 3.97 min as peak Y and of 4.74 min as peak X. The retention time of peak Y was the same as that of non-denatured cl-R339T (the relaxed form). It is striking that peak X increased faster than peak Y at early refolding times but then decreased as peak Y increased. In addition, Fig. 7b demonstrates that peaks X and Y contain both the heavy and light chains as does peak Z. These results together with our previous study (42) strongly suggest that peaks X and Y correspond to the pre-loop-inserted and loop-inserted cl-R339T, respectively.

Based on these results, we propose an extended pathway of Scheme 2 for the refolding of cl-R339T after the reassociation step as

$$
X \rightarrow Y \rightarrow Z
$$

where $X$ is the stressed state and $Y$ is the relaxed state. $k_f$ and $k_i$ are the first-order rate constants for the folding and the loop insertion, respectively. On the other hand, the refolding of cl-OVA can be expressed by a modified form of Scheme 2: $N(H/L) \rightarrow N(H/L)_{\text{relaxed}}$. Thus the relative areas of peaks X, Y, and Z at refolding time $t$, $A_x(t)$, $A_y(t)$, and $A_z(t)$ are given as

$$
A_x(t) = A_x(\infty) k_i \left\{ \exp(-k_f t) - \exp(-k_i t) \right\}/(k_i - k_f) \quad (Eq. 3)
$$

$$
A_y(t) = A_y(\infty) \left\{ k_f \exp(-k_f t) - k_i \exp(-k_i t) \right\}/(k_i - k_f) \quad (Eq. 4)
$$

$$
A_z(t) = A_z(\infty) \left\{ k_f \exp(-k_f t) - k_i \exp(-k_i t) \right\}/(k_i - k_f) \quad (Eq. 5)
$$

FIGURE 7. HPLC analysis for the stressed to relaxed transition during refolding. a, In$_N$-H-R339T (left) or In$_N$-H-OVA (right) was mixed with the light chain at 25 °C and allowed to refold at the same temperature for 1, 5, 15, or 30 min. The refolding was quenched by 5-fold dilution with a Tris-HCl buffer prechilled at 0.5 °C, and then the sample was analyzed by anion-exchange HPLC at 0.5 °C. The samples at refolding time 0 min were prepared by mixing of In$_N$-H and the light chain at 0.5 °C. For comparison, the non-denatured intact (int) and cleaved (cl) proteins were also analyzed. b, the samples collected in peaks X (lane 1), Y (lane 2), and Z (lane 4) at refolding time 5 min were assessed by Tricine SDS-PAGE. Non-denatured cl-R339T (lane 3) and cl-OVA (lane 5) were also loaded onto the gel. H and L represent the heavy and light chains, respectively, c, the relative areas of peaks X (triangles), Y (circles), and Z (diamonds) at various refolding times were plotted. The peak area of each non-denatured cleaved protein was defined as 1.0, and the solid curves were obtained from Equations 3, 4, and 5 in the text.
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FIGURE 8. FRET analysis of the stressed and relaxed forms. The Trp residues of the protein samples were excited at 295 nm in the presence of 0.1 mM ANS, and ANS fluorescence was recorded at 4 °C. The protein samples are: non-denatured int-OVA (solid black), cl-OVA (dashed block), int-R339T (solid red), cl-R339T (dashed red), and the proteins in peaks X (solid green), Y (solid blue), and Z (solid purple) in Fig. 7. Until mixing with ANS solution at 4 °C, these protein samples were kept at 0.5 °C or subjected to a thermal treatment (incubation at 25 °C for 1 h followed by cooling at 0.5 °C). The samples subjected to thermal treatment are represented by dotted curves of the same color.

\[ A_2(t) = A_2(\infty) \left\{ 1 - \exp(-k_t t) \right\} \quad (\text{Eq. 5}) \]

where \( A_2(\infty) \) and \( A_2(\infty) \) are the relative areas of peaks Y and Z at \( t = 120 \) min, respectively. As shown in Fig. 7c, the experimental data fitted well to these equations with kinetic parameters \( k_t' = 0.25 \pm 0.083 \text{ min}^{-1} \) and \( k_t = 0.15 \pm 0.068 \text{ min}^{-1} \) from Equation 3, \( k_f' = 0.24 \pm 0.071 \text{ min}^{-1} \) and \( k_f = 0.14 \pm 0.049 \text{ min}^{-1} \) from Equation 4, and \( k_p = 0.24 \pm 0.055 \text{ min}^{-1} \) from Equation 5. The obtained \( k_t \) and \( k_f \) values were almost exactly the same as the \( k_t \) values for the refolding of cl-OVA and cl-R339T in Table 1. The \( k_t \) value was similar to the first-order rate constant for the loop insertion of native R339T upon P1-P1 cleavage (0.24 min\(^{-1}\)) (28). These results are consistent with the refolding model that cl-R339T refolds into the relaxed serpin form via a stressed conformer.

FRET Analysis of the Stressed and Relaxed Forms—ANS is a useful indicator for the stressed or relaxed form in an ovalbumin mutant, R345A, that acquires the loop-inserting competence; the dye displays a higher binding capacity to the relaxed form than to the intact stressed form (43). Likewise the dye showed higher binding affinity to relaxed cl-R339T than to stressed int-R339T (see supplemental Fig. S2). When FRET measurements were carried out between Trp residues in ovalbumin as the donor and ANS as the acceptor, the fluorescence of non-denatured cl-R339T was higher than that of the stressed species (non-denatured int-OVA, cl-OVA, and int-R339T) (Fig. 8). This indicates that any of the high affinity ANS binding sites of the relaxed protein are near Trp residues: Trp\(^{148}\) in helix F, Trp\(^{184}\) in the top of strand 3A, and Trp\(^{267}\) in helix H (Fig. 1). To assess the conformational state of these regions, the proteins corrected in peaks X, Y, and Z (P\(_X\), P\(_Y\), and P\(_Z\)) were subjected to the FRET analysis. As shown in Fig. 8, the spectrum of P\(_X\) was very similar to that of non-denatured cl-R339T, whereas the FRET signals of P\(_Y\) and P\(_Z\) were as low as those of the stressed species. After incubation of the proteins for 1 h at 25 °C, the signal of P\(_X\) increased to 91% of that of non-denatured cl-R339T, whereas the spectra of P\(_Y\) and P\(_Z\) showed little change. These data show that the structures around the Trp residues of P\(_X\), P\(_Y\), and P\(_Z\) are well formed. In addition, P\(_X\) is substantially the same as the stressed form and capable of transforming to the relaxed state.

DISCUSSION

A central question related to serpins is how the polypeptide folds into a metastable state because it is key in an understanding of serpin functions typified by inhibition of proteases as well as in understanding serpin dysfunctions involving transition to the latent forms and polymers. It has been hypothesized that native serpins stay in a metastable intermediate state on the serpin folding pathway. Upon P1-P1’ cleavage, the metastable molecule can escape from the kinetic trap and then continue to fold into a more stable structure. A non-inhibitory serpin, ovalbumin, however, stays in the trap after P1-P1’ cleavage because of its bulky Arg\(^{339}\) at the proximal hinge (Fig. 1). This unique property raises a question: is cl-OVA able to form the relaxed structure through refolding? In a non-native state where the proximal and distal hinges, the breach, and the shutter should be unstrained, cl-OVA may adopt a more stable conformation upon refolding.

The present study clearly demonstrates that fully denatured cl-OVA refolds to the metastable stressed form with a \( T_m \) of 68.4 °C (Figs. 3 and 6). This indicates that ovalbumin peptide, although the RCL is unbound due to P1-P1’ cleavage, can attain the metastable fold from a fully denatured state. In contrast, urea-denatured cl-R339T refolded to the thermostable relaxed form with a \( T_m \) of 85.5 °C (Figs. 3 and 6), demonstrating that Arg\(^{339}\) at position P14 prevents refolding to the relaxed structure. The P14 residue of serpins is buried in a hydrophobic core of the protein upon insertion of the RCL (44); hence the failure of native cl-OVA to undergo the RCL insertion is explained by the difficulty in burying the bulky and charged residue Arg\(^{339}\) into the protein core. Our data show that Arg\(^{339}\) is not buried even by refolding, indicating that the protein core, the proximal hinge, and the breach are, at least partially, formed in an early refolding stage.

The refolding process of ovalbumin comprises the formation of an initial burst refolding intermediate involving local interactions followed by the refolding to the native state significantly involving non-local interactions (10, 17). The spectroscopic data (Fig. 2) and susceptibility to trypsin (Fig. 3c) show that the intermediate I\(_{\text{on}}\)-H is indistinguishable from the initial burst refolding intermediate of the intact protein. However, after the formation of I\(_{\text{on}}\)-H, conformational changes were not observed in the absence of the light chain (Fig. 2), indicating that the deletion of the region of the light chain is fatal for regaining structures involving non-local interactions. The light chain participates in strands 4B, 5B, and 1C in the native structure (Fig. 1) (45, 46). They are part of the B/C barrel believed to be formed at an early refolding stage of serpins as a folding nucleus (24). This nucleus is thought to be important for forming the
RCL in the stressed conformation because some mutations in the B/C region promote folding into uncleaved loop-inserted species (47). In the IN-H state, a folding nucleus is formed (Fig. 2, b and c), but its B/C region is truncated. The failure of IN-H to regain any structure may result from the truncated folding nucleus.

After mixing IN-H and the light chain, the refolding proceeded kinetically analogous to that of the intact protein (Fig. 5 and Table 1). This suggests that the correct folding nucleus is immediately formed after the mixing and then followed by the refolding through essentially the same pathways used by the intact protein. The disulfide exchange analysis (Fig. 4) endorses the immediate interactions in the B/C region. Cys367 and Cys382 of the light chain are in the B/C barrel in native ovalbumin (Fig. 1). The rapid increase of disulfide linkages involving Cys367 manifests the rapid contact between the heavy and light chains, and the practical absence of disulfide-involved Cys382 at early refolding times is consistent with the specific coordination of the light chain upon the interactions. Furthermore the cysteine residue initially attacked by Cys367 would be largely Cys11 in the B/C region of the heavy chain, highlighting the interaction between the two chains in the B/C region.

The final refolded products from cl-OVA and cl-R339T were the stressed and relaxed forms, respectively (Fig. 6). At what stage did the stressed to relaxed transition occur in the refolding of cl-R339T? The conformational analysis by anion-exchange HPLC provides the conclusion that the relaxed structure is formed after the formation of a stressed conformation. The HPLC data fitted well to the refolding model in Scheme 3, indicating that a parallel pathway for the direct folding N(H/L)stressed can be excluded. The consistency of the rate constants for the native folding kN and kN' supports that the folding step IN(H/L) → N(H/L)stressed of cl-R339T is essentially the same as IN(H/L) → N(H/L)stressed of cl-OVA. The value of kN' was almost consistent with the first-order rate constant for the RCL insertion of native R339T upon P1-P1' cleavage (0.24 min⁻¹) (28), suggesting that N'(H/L)stressed is equivalent to native stressed R339T.

The FRET analysis shows that the conformations around the Trp residues of N'(H/L)stressed are substantially the same as that of ovalbumin in the stressed state, meaning that the breach (Trp184 and helix F (Trp148)) of N'(H/L)stressed are well formed. This reveals the surprising refolding process of cl-R339T. Despite its unbound RCL, the central parallel sheet of strands 3A and 5A is formed, and helix F lies across the front of the sheet. After that, the hydrogen bonds between strands 3A and 5A are broken, helix F is unstrained and removed, and then the central antiparallel sheet of strands 3A, 4A (the RCL), and 5A is formed. In previous serpin studies, it has been believed that the connection between the P1 site and the B/C core plays a critical role for forming the serpin stressed structure. In the folding of intact serpins, it has been thought that the B/C core, which is connected to the RCL and formed at an early folding stage, hoists the RCL up, thereby allowing the formation of the less stable parallel sheet of strands 3A and 5A. In contrast, our results show for the first time that the connection is not associated with forming the stressed serpin structure but is crucial for maintaining it.

In conclusion, our model for the refolding process of cleaved ovalbumin (Fig. 9) is as follows. Upon refolding, the heavy chain refolds to an initial burst refolding intermediate, IN-H, with local interactions. Because of its truncated nature, IN-H is incapable of forming a correct folding nucleus and hence is incapable of further refolding. The light chain rapidly makes contacts with IN-H and then a reassociated intermediate, IN(H/L), is formed. IN(H/L) has the correct folding nucleus, essentially the same as that of the refolding intermediate of the intact protein, thereby resulting in the refolding into the stressed and cleaved forms of ovalbumin and R339T. It is striking that the P14 residue and the P1-P1’ bond are not associated with forming the stressed structure but are crucial for maintaining it. Instead intramolecular interactions in IN are crucial for the folding to the stressed conformation. Unless the interactions are disrupted, the protein is on the refolding pathway to the stressed

![Schematic diagram of the refolding process of cleaved ovalbumin](image.png)
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form. Or vice versa, disrupting the interactions, as several serpin studies indicate (47–49), will result in misfolding or taking short cut pathways to adopt alternative serpin conformations including latent species and polymers.

After the formation of the stressed conformation, cl-R339T further refolds to the relaxed structure. This step comprises partial unfolding of refolded helix F and breaking the hydrogen bonds between strands 3A and 5A followed by the formation of the central antiparallel sheet including the RCL and restoration of helix F (48, 50). Surprisingly although the RCL is unbound, the central antiparallel sheet is not directly formed from the refolding intermediate state where helix F and strands A are partially unfolded (48, 50, 51). The antiparallel sheet is formed via a detour that includes formation and subsequent split of the parallel sheet of 3A and 5A. This indicates that the intramolecular interactions in the stressed conformation are crucial for forming the relaxed structure. That is to say, the stressed form of serpins is a non-productive intermediate that is trapped within the inevitable detour in the folding pathway to the relaxed form. Such a folding intermediate has not been reported in other proteins. Nevertheless this folding mechanism may be a key to understanding great conformational rearrangements of proteins in regard to conformational diseases.

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