Ovalbumin, a member of the serpin superfamily, contains one cystine disulfide (Cys73-Cys120) and four cysteine sulfhydryls (Cys11, Cys30, Cys367, and Cys382) in the native state. To investigate the folding mechanism of ovalbumin, a urea-denatured disulfide isomer with a mispaired disulfide Cys367-Cys382 (D[367–382]) and its derivative (D[367–382/CM-73]) in which a native cystine counterpart of Cys73 is blocked by carboxymethylation were produced. Both the denatured isomers refolded within an instrumental dead time of 4 ms into an initial burst intermediate Iₐ with partially folded conformation. After the initial burst phase, most of the D[367–382] molecules further refolded into the native form. In contrast, upon dilution of D[367–382/CM-73] with the refolding buffer, the protein stayed in the Iₐ state as a stable form, which displayed a partial regain of the native secondary structure and a compact conformation with a similar Stokes radius to the native form. The structural characteristics of Iₐ were clearly differentiated from those of an equilibrium intermediate Iₐ that was produced by dilution with an acidic buffer of urea-denatured ovalbumin; Iₐ showed much more hydrophobic dye binding and a larger Stokes radius than the Iₐ state, despite their indistinguishable far-UV circular dichroic spectra. The non-productive nature of Iₐ highlighted the importance of a compact conformation of the Iₐ state for subsequent native refolding. These observations were consistent with a refolding model of ovalbumin that includes the regain of the partial secondary structure and of the compactness of overall conformation in an initial burst phase before the subsequent native refolding.

The importance of protein folding studies has been increasing, because they give important information about the molecular mechanism for novel types of disease that involve a protein aggregation process (1–5). The serpins, a group of serine proteinase inhibitors, are an interesting target for investigation of the protein folding mechanism. They undergo unique conformational change upon exerting the inhibition activity; after receiving cleavage at the canonical P1-P1' site by a target proteinase, the reactive center loop is inserted into the central β-sheet A (6). This dynamic conformational change accompanies a great structural stabilization and has been considered to be from a metastable state into a fully stabilized state (7). The folding analysis of serpins should therefore explain how a protein molecule folds into a metastable state with native protein nature. The folding process of serpins has, however, been poorly understood. This is largely related to the large protein size with a molecular mass of around 40 kDa, which makes it difficult for the application of powerful protein folding analysis, such as nuclear magnetic resonance.

Ovalbumin, despite its non-inhibitory nature (8), is a member of the serpin superfamily, because of a close similarity in the primary and tertiary structures (9–11) and the specific loop cleavage at the canonical P1-P1’ site by a serine proteinase (12). Furthermore, recent thermodynamic and crystallographic evidence from our laboratory has clearly demonstrated that ovalbumin exerts, by the replacement of a single hinge residue, the inherent metastable nature that undergoes structural transition into the loop-inserted, thermostabilized form following P1-P1’ cleavage (13). Ovalbumin should be, therefore, a competent model for the analysis of the metastable folding mechanism. We have shown that intramolecular sulfhydryl/disulfide exchange reactions are a useful probe for the analysis of the unfolding and refolding process of ovalbumin (14–19). Ovalbumin consists of a single polypeptide chain of 385 amino acid residues and contains 6 cysteine residues of Cys11, Cys30, Cys73, Cys120, Cys367, and Cys382; only Cys73 and Cys120 form a disulfide bond in the native state (20, 21). Under highly denaturing conditions, the egg white protein undergoes extensive sulfhydryl/disulfide exchanges producing all the 15 possible disulfide isomers with one disulfide and four sulfhydryls (14). Thus the disulfide isomers are distributed at equilibrium depending on loop length (the number of amino acid residues separating the two cysteines) to a power of about ~2. From the complex denatured state, most of the ovalbumin molecules refold into the native form with Cys73-Cys120 through intrachain sulfhydryl/disulfide exchanges (15). This enables us to investigate the refolding processes from different disulfide isomers with different conformational entropy. That the native cystine disulfide is regained without the help of an added chemical oxidant implies that the sulfhydryl accessibility problem (22, 23), inherently inevitable in the oxidative refolding system, can be circumvented. As an alternative advantage, a possible folding intermediate can be trapped in a stable form under native folding conditions, as demonstrated here, when one of the cysteine residues participating in the native disulfide bond is blocked.

In a previous study using a disulfide isomer (16), we have demonstrated that the refolding process of ovalbumin includes a non-productive pathway as displayed in Scheme 1. When the urea-denatured isomer with the native disulfide Cys73-Cys120...
(D[73–120]),1 produced by protein incubation under acid/urea conditions, is diluted into a near neutral folding buffer, ovalbumin forms an initial burst intermediate I\(_N\)[73–120]. This intermediate can correctly refold into the native form N[73–120] with a first-order rate constant \(k_f\) of about 0.2 min\(^{-1}\). At the same time, however, the intermediate I\(_N\)[73–120] undergoes reversible intrachain sulfhydryl/disulfide exchanges, upon generating the mispaired disulfide intermediate pool, I\(_N\)[mis-SS]. In the present study, we have prepared alternative denatured disulfide isomers: the disulfide isomer with mispaired Cys\(^{367}\)-Cys\(^{382}\) (D[367–382]) and its derivative (D[367–382/CM-73]) in which a counterpart cysteine of the native disulfide, Cys\(^{382}\), is blocked by \(S\)-carboxymethylation. Here we demonstrate that the mispaired disulfide isomer D[367–382] can correctly refold into N[73–120] through disulfide rearrangements. In contrast, D[367–382/CM-73] stays in the intermediate I\(_N\) state under the native refolding conditions. The stable nature of the intermediate enabled us to analyze its conformational characteristics by several conventional approaches.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ovalbumin (\(\varepsilon\)-ovalbumin, diprophosphorylated form) was purified from fresh egg white by crystallization in an ammonium sulfate solution and subsequent ion exchange column chromatography as described previously (24, 25). Native cystine-reduced ovalbumin, N[SH], was prepared by incubation of ovalbumin (20 mg/ml) at 37 °C for 2 h with 15 mM DTT in buffer B (50 mM Tris-HCl buffer, pH 8.2, containing 1 mM sodium EDTA). N(CM-73) was prepared by incubation of N[SH] at 37 °C for 10 min with 40 mM IAA in buffer B. Under the conditions, the sulphydryl of Cys\(^{37}\) was specifically carboxymethylated in N[SH] with a yield of 99%. The conformational state of N(CM-73) was almost indistinguishable from disulfide-intact ovalbumin as evaluated by far-UV CD and intrinsic tryptophan fluorescence spectra and trypsin resistance analyses (data not shown).

**Diphenylcarbamoyl chloride-treated trypsin** (type XI), chymotrypsin (type II), rabbit muscle lactate dehydrogenase (type XI), human trypsin (grade II), and bovine serum albumin were purchased from Sigma. Bovine liver catalase and bovine erythrocyte carbonic anhydrase were purchased from Nakalai Tesque. *Achromobacter protease I* (EC 3.4.21.50) was obtained from Wako Pure Chemical.

**Preparation of Urea-denatured, Mispaired Disulfide Isomers**—The outline of preparation of the urea-denatured mispaired disulfide isomers D[367–382] and D[367–382/CM-73] is shown in Fig. 1. Step 1 was to produce a mixed-disulfide protein derivative; native ovalbumin (20 mg/ml) was incubated at 25 °C for 1 h with 2 mM Pyr-S-S-Pyr in buffer A (50 mM potassium phosphate buffer, pH 2.2, containing 1 mM sodium EDTA), then excess Pyr-S-S-Pyr and Pyr-SH were removed by gel filtration using a Sephadex G-10 column (Amersham Biosciences, NAP-10) equilibrated with buffer B. In Step 2, the native cystine disulfide Cys\(^{37}\)-Cys\(^{382}\) of the derivative was reduced by incubation at 37 °C for 4 h with 15 mM DTT in buffer B, then the sample was passed through a Sephadex G-10 column equilibrated with buffer A. The urea-denatured, mispaired protein D[367–382] was produced in Step 3 by incubation of the cystine-reduced, mixed-disulfide derivative at 37 °C for 30 min with 6 M urea in buffer A; during this incubation, a mispaired disulfide Cys\(^{37}\)-Cys\(^{382}\) and Pyr-SH are produced by the attack of the Cys\(^{382}\) sulphydryl against the mixed-disulfide of Cys\(^{37}\) (19).

Another urea-denatured mispaired disulfide isomer, D[367–382/CM-73] was prepared in the same way except that the Cys\(^{37}\) sulphydryl was carboxymethylated, prior to Step 3, by incubation of the cystine-reduced, mixed-disulfide derivative with 40 mM IAA at 37 °C for 10 min. Disulfide-reduced, urea-denatured proteins DISH) and D(CM-73) were prepared by incubation of D[367–382] and D[367–382/CM-73] with 5 mM DTT at 37 °C for 30 min in buffer B containing 9 M urea, respectively.

**Refolding of the Urea-denatured Proteins**—Refolding was initiated at 25 °C by 20-fold dilution of the urea-denatured proteins with buffer C (50 mM Tris-HCl buffer, pH 8.6, containing 1 mM sodium EDTA) giving a final pH value of 8.2. The proteins were allowed to refold at 25 °C. An equilibrium intermediate I\(_N\) was produced by 20-fold dilution of the urea-denatured proteins with buffer A. The buffers were degassed at reduced pressure and equilibrated under N\(_2\) atmosphere prior to the refolding and contained 0.5 mM DTT for the experiments for the disulfide-reduced proteins.

**Analysis for Disulfide-involved Cysteines**—Disulfide-involved cysteines were determined by a peptide mapping analysis as described previously (14). Briefly, the urea-denatured protein in acidic pH conditions was neutralized by addition of 1 M Tris solution and alkylated with 0.1 mM iodoacetamide in the presence of 9 M urea. The alkylated protein was precipitated in a cold acetone-HCl solution, dissolved in buffer B containing 8 M urea, reduced with DTT, and then modified with a fluorescent alkylation reagent, N-iodoacetyl-N'-((5-sulfo-1-naphthyl)-ethylenediamine. The modified protein was extensively proteolized with combinations of trypsin, chymotrypsin, and *Achromobacter protease* I. The resultant peptides were analyzed by reversed-phase high performance liquid chromatography, and the modified cysteine peptides were detected by fluorescence (excitation, 340 nm; emission, 320 nm).

For the refolding analysis, sulphydryl/disulfide exchanges were quenched at various refolding times by mixing the protein samples with a 0.24 volume of 2 M HCl. Disulfide-involved cysteines were determined in the same way.

**Intrinsic Tryptophan Fluorescence and Far-UV CD Spectrum**—The fluorescence spectrum of ovalbumin was measured with a fluorescence spectrophotometer (Hitachi, model F-3000). The intrinsic tryptophan fluorescence in ovalbumin was excited at 285 nm, and emission spectrum was recorded at a wavelength range from 300 to 420 nm. All measurements were carried out at a constant temperature of 25 °C.

For the spectrum measurements at an early refolding time, a stopped-flow reaction analyzer (Applied Photophysics Ltd., UK) was employed. The time course of fluorescence intensity changes was monitored at various emission wavelengths (excitation wavelength, 295 nm), and the data at a refolding time of 5 ms were plotted. For the control experiments, the native ovalbumin and the urea-denatured proteins were diluted, respectively, by the refolding buffer (buffer C) containing 0.82 M urea and by 0.25 M HCl containing 9 M urea and 1 mM sodium EDTA (pH 2.2), and the fluorescence intensity at 338 nm was recorded in the same way. The averaged data of 10–15 traces were obtained. The dead time for mixing was determined to be 4 ms by a model reaction between 2,6-dichlorophenolindophenol and I-ascorbate (26).

The far-UV CD spectrum was recorded at 25 °C with a spectropolarimeter (JASCO, J-720). The CD data were expressed as mean residue ellipticity (deg cm\(^2\)/dmol) by using 111 as the mean residue weight of ovalbumin. CD spectra at a short refolding time were determined by measuring the time-dependent increase in CD ellipticities at various wavelengths, and the values at the 10-s refolding time were plotted as a function of the wavelength. The averages of eight time traces were determined.

The time course of the refolding after the initial burst phase was monitored by the CD ellipticity at 222 nm and the intrinsic tryptophan fluorescence excited at 295 nm. The data were obtained as the averages of triplicate determinations. The fraction of the native form at the refolding time of \(t\) \(F\(_N\)(t)\) was calculated by using the equation: \(F\(_N\)(t) = (X\(_N\) - X\(_N\)\_0)/X\(_N\)\_0 - X\(_I\)\_0\), where \(X\(_N\)\_0\) and \(X\(_I\)\_0\) are the initial values and the values at the refolding time \(t\), respectively. For the CD and fluorescence
The regain of the native conformation at near neutral pH, an alkylation reagent, such as 2,2-dimethoxypropane, is used to specifically react with the Cys367 sulfhydryl at pH 2.2 (19). By use of the reaction, a mixed-disulfide protein derivative was produced in Step 1. As Step 2, the native cystine Cys73-Cys120 in the native protein was reduced with DTT at neutral pH, generating the cystine-reduced, mixed-disulfide derivative. In the presence of a high concentration of urea at pH 2.2, Pyr-SH is released from the mixed disulfide derivative by the nucleophilic attack of the nearest cysteine residue in the primary structure, Cys382 (19). Thus the mispaired disulfide isomer D[367–382] was estimated to be more than 97%.

The other urea-denatured mispaired disulfide isomer D[367–382/CM-73] was prepared by placing the cystine-reduced, mixed-disulfide derivative in acidic urea denaturing conditions as Step 3. The specific disulfide formation between Cys367 and Cys382 in the urea-denatured sample was confirmed by a peptide mapping analysis as shown in Fig. 2A; this figure clearly shows that almost all of the disulfide-forming cysteines consisted of Cys367 and Cys382. By comparison with the overall labeling analysis that leads to the detection of all the six cysteines (Fig. 2B), the yield of D[367–382] was estimated to be more than 97%.

The rate of temperature change was 1 K/min. The data were obtained as the averages of eight triplicate determinations. The yields of D[367–382] and D[367–382/CM-73] were prepared as summarized in Fig. 1. Ovalbumin takes a highly ordered molten-globule state at acidic pH, and Pyr-S-S-Pyr reacts specifically with the Cys367 sulfhydryl at pH 2.2 (19). By use of the reaction, a mixed-disulfide protein derivative was produced in Step 1. As Step 2, the native cystine Cys73-Cys120 in the native protein was reduced with DTT at neutral pH, generating the cystine-reduced, mixed-disulfide derivative. In the presence of a high concentration of urea at pH 2.2, Pyr-SH is released from the mixed disulfide derivative by the nucleophilic attack of the nearest cysteine residue in the primary structure, Cys382 (19). Thus the mispaired disulfide isomer D[367–382] was prepared by placing the cystine-reduced, mixed-disulfide derivative in acidic urea denaturing conditions as Step 3. The specific disulfide formation between Cys367 and Cys382 in the urea-denatured sample was confirmed by a peptide mapping analysis as shown in Fig. 2A; this figure clearly shows that almost all of the disulfide-forming cysteines consisted of Cys367 and Cys382. By comparison with the overall labeling analysis that leads to the detection of all the six cysteines (Fig. 2B), the yield of D[367–382] was estimated to be more than 97%.

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The refolding under disulfide-reduced conditions was therefore employed as a control experiment in the present refolding study. The starting samples of the urea-denatured disulfide-reduced forms D[SH] and D[CM-73] were prepared by reduction with DTT of the disulfide-bonded counterparts of D[367–382] and D[367–382/CM-73], respectively.

Refolding Detected by Optical Methods—The conformational change during the ovalbumin refolding was examined by intrinsic tryptophan fluorescence, far-UV CD spectrum and ANS binding analyses. As shown in Fig. 3 (A and B), all the urea-denatured proteins showed typical fluorescence and CD spectra for the unfolded protein; essentially no spectrum difference was detected among the different urea-denatured proteins. The native ovalbumin and the urea-denatured proteins displayed the maximum fluorescence at 338 and 352 nm, respectively, and the maximum fluorescence intensity of the urea-denatured proteins was about 33% of that of the native ovalbumin.

We have previously shown that two types of partially folded intermediates IN and IA are formed within an initial burst phase during the refolding of urea-denatured ovalbumin with the correct disulfide (D[73–120]) (16); the intermediate IN, produced by dilution of D[73–120] with a near neutral buffer, can refold to the native protein, but IA, produced by dilution of the same urea-denatured protein with an acidic buffer, stays in the partially folded state without being transferred to a near neutral pH condition in which the protein refolds correctly in the same way. In the present study, the equivalent intermediates IN and IA were found to be formed during the refolding of the urea-denatured, mispaired disulfide isomers and of their disulfide-reduced counterparts, as stated below.

Ovalbumin contains three tryptophan residues, Trp148 in helix F, Trp184 as the nearest neighbor residue of the carboxyl terminus of strand 3A, and Trp267 in helix H (9–11). Upon rapid stopped-flow dilution with a near neutral pH buffer, the urea-denatured, mispaired disulfide protein D[367–382] refolded within the mixing dead time of 4 ms into an initial burst state that showed at 338 nm an intermediate intrinsic tryptophan fluorescence intensity between the native and denatured forms (Fig. 3A, inset). The same experiment was done with the different urea-denatured proteins at various wavelengths of fluorescence emission. When the fluorescence intensity at 5 ms after the rapid mixing was plotted as a function of the wavelength, the fluorescence spectrum obtained was almost exactly the same for all the intermediates produced from the different urea-denatured proteins; the spectrum of the initial burst intermediate was the one with the same peak wavelength (338 nm) but with much less intensity (56%) as compared with that of the native protein (Fig. 3A).

The conformational state of the early refolding intermediate was also analyzed by far-UV CD spectrum analysis. In this analysis, the CD ellipticities at the refolding time of 10 s after a manual mixing were determined at various wavelengths. As shown in Fig. 3B, the CD spectra at 10-s refolding were almost indistinguishable among the intermediates from the different urea-denatured proteins and displayed about 58% of the absolute value of the native CD ellipticity at 222 nm. When the same manual mixing experiment was carried out by the intrinsic tryptophan fluorescence analysis, the obtained fluorescence spectra at the refolding time of 10 s were almost indistinguishable from those of the initial burst intermediates at 0.5 ms refolding (data not displayed). This was reasonably accounted for by a slow rate of refolding after the initial burst intermediate formation; the most rapid refolding from the initial burst intermediate occurs for the disulfide-reduced forms (D[SH] and D[CM-73]) with the first-order rate constant of 0.2 min⁻¹ (Fig. 4A), indicating that the formation of the native form should be

![Fig. 2. Peptide-mapping analysis for the disulfide isomers.](image-url)

The disulfide-involved cysteines were analyzed for D[367–382] (A) and D[367–382/CM-73] (C) as described in the text. In B and D, the overall labeling experiments by which all the cysteine residues are alkylated with a fluorescent probe were done for D[367–382] and D[367–382/CM-73], respectively. The numbers above the peaks represent the residue numbers of the labeled cysteines.

as IAA, is inaccessible to the sulphydryls of Cys11, Cys30, Cys367, and Cys382, but Cys73 and Cys120 can be readily alkylated in their reduced state with IAA (17). In the present study, we found that the Cys73 sulphydryl is more reactive to IAA than the Cys120 sulphydryl and that, in an optimized condition, Cys73 can be specifically carboxymethylated. As shown in Fig. 2D, when the cystine-reduced, mixed-disulfide derivative was carboxymethylated with 40 mM IAA at pH 8.2 (producing the carboxymethylated, mixed-disulfide derivative) and then subjected to the overall labeling analysis, the Cys73 sulphydryl was found to be almost completely blocked, but no other cysteine sulphydryls were carboxymethylated with IAA. The carboxymethylated, mixed-disulfide derivative was then denatured during Step 3, and the sample was analyzed for the disulfide-forming cysteines by the peptide mapping method. As shown in Fig. 2C, the disulfide-forming cysteines comprise almost totally Cys367 and Cys382.

Ovalbumin can also refold correctly without any intrachain disulfide through an equivalent intermediate IN state (16); the absence of the interconversion of IN with I[miSS] pool results in a more straightforward refolding of ovalbumin (16).
Fig. 3. Intrinsic tryptophan fluorescence (A), far-UV CD spectrum (B), and ANS binding (C) analyses. The spectra shown are: the native ovalbumin (N[73–120], thick solid line), the urea-denatured disulfide isomers (D, dashed lines), the proteins refolded for 20 h from the urea-denatured disulfide isomers (20H, dotted lines), and the equilibrium intermediate state I_A produced by dilution of the urea-denatured disulfide isomers with acidic buffer (I_a, thin solid lines). In C, the fluorescence of ANS was almost undetectable in the presence of the native proteins (N) and of the urea-denatured proteins (D). The attached lowercase italic letters on the right side of D represent the disulfide type (a, D[367–382]; b, D[367–382]/CM-73; c, D[SH]; and d, D[CM-73]) and are arranged from the top to the bottom according to the fluorescence intensity at the peak wavelength or the absolute value of CD ellipticity. At a prolonged refolding time of 20 h, the fluorescence intensity at 338 nm reached 97% of the values for the native protein, respectively (Fig. 3, A and B). The refolded protein from D[367–382]/CM-73, however, did not show any increase in the fluorescence intensity and CD ellipticity after the initial burst phase (Fig. 3, A and B).

When the urea-denatured proteins were refolded by dilution with an acidic buffer, they showed fluorescence spectra with 94% of the native intensity at the peak wavelength of 338 nm.

3.4% only at the refolding time of 10 s. The far-UV CD spectra obtained at the refolding time of 10 s (Fig. 3B) should be, therefore, essentially the same as the ones for the initial burst intermediates. The conformational characteristics of the initial burst intermediate, obtained by the fluorescence and far-UV CD spectra, were consistent with those of I_N produced during the refolding from the urea-denatured, correct disulfide protein D[73–120] (16).

After the formation of the initial burst intermediate I_N, all the urea-denatured proteins except for D[367–382]/CM-73 slowly refolded as reflected in the increases of the fluorescence intensity and of the absolute value of CD ellipticity. At a prolonged refolding time of 20 h, the fluorescence intensity at 338 nm and the CD ellipticity at 222 nm reached 97 and 90% of the values for the native protein, respectively (Fig. 3, A and B). The refolded protein from D[367–382]/CM-73, however, did not show any increase in the fluorescence intensity and CD ellipticity after the initial burst phase (Fig. 3, A and B).

Fig. 4. The time course of the refolding after the initial burst intermediate formation. In A, the urea-denatured disulfide isomers D[367–382] (a), D[367–382]/CM-73 (b), D[SH] (c), and D[CM-73] (d) were refolded at pH 8.2 and 25 °C, and the time-dependent conformational regain was monitored by the CD ellipticity at 222 nm (pluses), the intrinsic tryptophan fluorescence at 338 nm (closed circles), and the trypsin resistance (open diamonds). The ordinate shown by F(t) represents the fraction of the native form at the refolding time of t, calculated as described in the text. In B, the urea-denatured proteins D[367–382] (a: crosses), D[367–382]/CM-73 (b: open squares), D[SH] (c: open triangles), and D[CM-73] (d: open circles) were refolded in the same conditions, and the time-dependent decrease of ANS binding was measured. The abscissa represents the sum of the time for refolding and in incubation with ANS. For both panels, the solid line b represents linear least-squares fits to the experimental data. The solid curves for the disulfide-reduced proteins c and d represents single-exponential least-squares fits to the experimental data. For the refolding of D[367–382], double-exponential least-square fits to the experimental data are shown as the solid curve a.
The CD spectrum of the acidic intermediate was essentially the same as that of IN, showing about 56% of the native CD ellipticity at 222 nm (Fig. 3B). The fluorescence intensity and the absolute value of the CD ellipticity did not increase after the initial burst phase in this acidic condition (data not shown). These spectral characteristics of the acidic intermediate were consistent with those of the previous equilibrium intermediate IN produced by dilution of D[73–120] with the same acidic buffer (16).

The results from the intrinsic tryptophan fluorescence and far-UV CD spectra show that the initial burst intermediate IN is formed during the refolding from D[367–382], D[367–382/CM-73], D[CM-73], and D(SH) as well as from D[73–120] with an equivalent conformational state. The same is also true for the acidic equilibrium intermediate IA; the conformational state of this intermediate is essentially the same for the proteins produced from the different disulfide forms.

The conformational difference between the two intermediates of IN and IA was detected by ANS binding analyses. Fig. 3C shows the emission spectra of ANS in the presence of various states of ovalbumin. The ANS binding was almost undetectable for the native or urea-denatured ovalbumin. In contrast, greatly increased fluorescence emission with a peak at 472 nm was observed in the presence of the intermediate IA for any disulfide types. The IA state produced by refolding from D[367–382/CM-73] showed a much lower level of ANS binding. The results from the ANS binding analyses were consistent with the view that the intermediate IA is a more extended molecule with an exposed hydrophobic core than the intermediate IN.

Time Course of Refolding after the Initial Burst Phase—The time course of refolding after the initial burst phase was examined by trypsin resistance, far-UV CD, intrinsic tryptophan fluorescence, and ANS binding analyses (Fig. 4). The trypsin resistance assay is a sensitive and reliable probe for the refolding analysis, because ovalbumin is highly resistant against the protease in the native conformation (17), because of the non-basic nature of the P1 residue (alanine residue) (7).

Fig. 4A shows that the time courses of the refolding from D[367–382], D[367–382/CM-73], D(SH), and D(CM-73) were almost exactly the same for the three former conformational probes. The time courses were also consistent with the decrease of the ANS binding (Fig. 4B).

The time course of the refolding from D[367–382] showed a biphasic progress curve (the sum of two exponentials). Such biphasic kinetics was consistent with the native refolding of IN[73–120] and the involvement of disulfide rearrangements in the IN state. In contrast, both D(SH) and D(CM-73) refolded with much accelerated rates and with simple monophasic kinetics; the first order rate constants determined by the trypsin resistance, CD, fluorescence, and ANS binding analyses were the same value of 0.2 min$^{-1}$ for the refoldings from D(SH) and D(CM-73). This can be accounted for by the absence of the disulfide rearrangements in the IN state (IN[SH] or IN[CM-73]) that is competent for the subsequent folding into the native disulfide-reduced form N(SH). The same monophasic refolding kinetics for D(CM-73) and D(SH) indicate that the kinetic process of refolding under the disulfide-reduced conditions is not significantly affected by alkylolation of the Cys$^{19}$ sulfhydryl.

One of the most striking observations in Fig. 4 was related to the refolding process from D[367–382/CM-73]. The trypsin resistance analysis revealed the inability of D[367–382/CM-73] to refold natively. Furthermore, the results from the CD, fluorescence, and ANS binding analyses demonstrated that ovalbumin refolded from D[367–382/CM-73] stays in the IN state.

Differential Scanning Calorimetry Analyses—The preceding data indicate that most, if not all, of the D[367–382] and D(CM-73) molecules refolds into the native state in a prolonged incubation time (Figs. 3 and 4). The integrity of native refolding was investigated more rigorously by differential scanning calorimetry. As shown in Fig. 5, the protein refolded for 20 h from D[367–382] showed the thermal transition at almost the same melting temperature ($T_m$, 76.7 °C) as the disulfide-bonded native protein ($T_m$, 77.0 °C). No significant difference was detected among the disulfide-reduced native proteins of N(SH) ($T_m$, 70.7 °C) and N(CM-73) ($T_m$, 70.1 °C) and the disulfide-reduced, refolded proteins from D(SH) ($T_m$, 70.8 °C) and D(CM-73) ($T_m$, 70.0 °C). The results from the differential scanning calorimetry therefore reinforced the correct refolding of ovalbumin from D[367–382], D(SH), and D(CM-73).

In contrast, the protein refolded from D[367–382/CM-73] did not show any clear thermal transition peak. This was consistent with the absence of the native refolding after the IN state (Figs. 3 and 4) for the protein refolded from D[367–382/CM-73]. A slight bulge detected at around 70 °C may be related to a possible contamination of D(CM-73) in the D[367–382/CM-73] preparation.

Sulfhydryl/Disulfide Exchanges during the Refolding—We have previously demonstrated that ovalbumin undergoes disul-
fide rearrangements by intrachain sulfhydryl/disulfide exchange reactions in the initial burst intermediate state \( I_N \) as shown in Scheme 1 (16). This suggests the inclusion of intrachain sulfhydryl/disulfide exchanges in the \( I_N \) state during the refolding from the urea-denatured, mispaired disulfide isomer D[367–382]. The disulfide-involved cysteines were determined at various refolding times by the peptide-mapping analysis. As shown in Fig. 6A, Cys120 and Cys382, which were detected as only the disulfide-involved cysteines at the refolding time 0 decreased immediately and continuously after the initiation of the refolding; the decreases in these non-native cysteines accompanied the increases in the other four cysteines of Cys367, Cys382, Cys73, and Cys120. The disulfide-involved Cys73 and Cys120 both increased continuously, and their amounts were estimated to be about 80% at 20 h of the refolding. In contrast, the increases in Cys11 and Cys30 were only tentative, followed by gradual decreases with time of refolding. The data were consistent with the view that most, if not all, of the urea-denatured, mispaired disulfide isomer can refold to the native disulfide form through intrachain sulfhydryl/disulfide exchange reactions.

The intrachain sulfhydryl/disulfide exchanges also occurred when D[367–382/CM-73] was transferred into the refolding buffer. As shown in Fig. 6B, Cys367 and Cys382 detected as disulfide-involved cysteines decreased after the transfer into the refolding buffer, whereas Cys11, Cys30, and Cys120 increased. It was confirmed that Cys73 did not increase as a disulfide-involved cysteine because of its blocked sulfhydryl nature; this situation resulted in a limited level as disulfide-involved cysteine for Cys120, which is the counterpart of the native disulfide pairing. The distribution of the various disulfide isomers reached equilibrium at 20 h; the amounts of disulfide-involved cysteines were about 47% for Cys11, Cys30, and Cys120 and about 27% for Cys367 and Cys382.

Size-exclusion Chromatography of Various States of Ovalbumin—The preceding data (Figs. 3–5) strongly suggest that ovalbumin refolded from D[367–382/CM-73] stays in the initial burst intermediate state with a partially folded conformation. As an alternative intermediate, ovalbumin assumes a partially folded conformation with the same far-UV CD spectrum as \( I_N \) state upon dilution of the urea-denatured proteins with an acidic buffer (Fig. 3). The Stokes radii were determined by size-exclusion chromatography for various ovalbumin states, including \( I_N \) and \( I_A \). As shown in Table I, the differential disulfide structures did not affect the Stokes radius for either native (30.3–31.0 Å), \( I_N \) (38.6–39.4 Å), or urea-denatured (58.2–59.5 Å) states. The protein refolded from D[367–382/CM-73] was a compact molecule with a Stokes radius of 31.6–33.5 Å, which was close to the value for the native protein. The acidic equilibrium intermediate \( I_N \) had a less compact conformation with a Stokes radius of about 39 Å, although it was less extended than the urea-denatured protein.

**DISCUSSION**

The present study demonstrates the usefulness, for the analysis of a large protein, of a refolding system that includes intrachain disulfide rearrangements. A urea-denatured, mispaired disulfide isomer D[367–382] of ovalbumin was found to spontaneously refold via an initial burst intermediate \( I_N \) into the native form N[73–120], as evidenced by a variety of conformational probes. More importantly, D[367–382] refolded into the state with the native thermostability, indicating the metastable refolding of ovalbumin in vitro. The obtained data, along with our previous ones (14–18), demonstrate for the first time the serpin refolding mechanism from the fully denatured state and should provide important information about how the protein folds into a metastable state.

Model Presentation for the Ovalbumin Refolding—Fig. 7 summarizes the overall refolding process of ovalbumin as an extended form of Scheme 1. Ovalbumin undergoes extensive sulfhydryl/disulfide exchanges under highly denaturing and near neutral pH conditions as shown by the arrows with dotted lines, producing all the 15 possible disulfide isomers with one disulfide and four sulfhydryls (14); the disulfide isomers distribute at equilibrium depending on loop length (the number of amino acid residues separating the two cysteines) to a power of about –2. From this complex denatured state, about 80% of the ovalbumin molecules refold into the native form through extensive intrachain sulfhydryl/disulfide exchanges (15). The 14 mispaired disulfide isomers can be categorized into two classes; class I comprises the eight isomers ([mis-SS]1 pool: [11–73], [30–73], [73–367], [73–382], [11–120], [30–120], [120–367], and [120–382]) in which one of the two cystine-forming cysteines is a native one, and class II contains the six isomers ([mis-SS]2 pool: [11–30], [11–367], [11–382], [30–367], [30–382], and [367–382]) in which both the disulfide-forming cysteines are non-native. The present data clearly demonstrate...
that a denatured class II disulfide isomer can refold into the native state.

A previous kinetic study of ovalbumin refolding (16) has shown that the urea-denatured native disulfide isomer D [73–120] forms an initial burst intermediate IN[73–120] upon dilution into a near neutral folding buffer. This intermediate refolds correctly into the native form N[73–120] with a first order rate constant of 0.2 min⁻¹ as shown by the open arrows. At the same time, however, IN[73–120] undergoes irreversible intrachain sulfhydryl/disulfide exchange reactions that generate the mispaired disulfide intermediate IA[73–120] (Scheme 1). The present finding that the mispaired disulfide Cys367-Cys382 is rearranged toward the native pairing of Cys73-Cys120 (Fig. 6) can be therefore accounted for by the occurrence of intrachain sulfhydryl/disulfide exchange reactions in the initial burst phase IN, which produces the folding competent intermediate IN[73–120] as the reverse reaction. The IN intermediate produced from D[367–382]CM-73 was found to lack the correct folding ability into the native form (Figs. 3 and 4) despite its potential to be converted into a variety of disulfide isomers. This reinforces that IN[73–120] is the only competent intermediate for the subsequent correct refolding under the disulfide-bonded conditions (16).

The model shown in Fig. 7 also includes the refolding process under the disulfide-reduced conditions. In contrast to the disulfide-bonded refolding, disulfide-reduced ovalbumin can refold in a straightforward way to the native form N[SH] with essentially the same first order rate constant of 0.2 min⁻¹ as the true folding rate constant from IN[73–120] to N[73–120] (16). This leads to the conclusion that the native disulfide Cys73-Cys120 is not necessary for native refolding, but a mispaired disulfide prevents the intermediate IN from the correct refolding. In other words, Cys73-Cys120 is just acceptable rather than crucial for the native conformation. In the native state, the redox conversion between N[73–120] and N[SH] is readily brought about without any conformational distortion in the presence of a reduced or oxidized form of a sulfhydryl reagent (17).

Refolding analyses under acidic conditions also provide useful information. When urea-denatured ovalbumin (D[73–120]) is diluted with an acidic buffer, pH 2, it is transformed into a partially folded conformational state IA that has the same far-UV CD spectrum as the IN state. Native ovalbumin retains its ordered conformational characteristics at this pH value (18, 29). Under the acidic conditions, complex sulfhydryl/disulfide exchange reactions are completely blocked. The intermediate IN[73–120], however, cannot refold directly into N[73–120] unless it is transferred to a near neutral pH buffer where the protein refolds correctly through the IA state (16). The absence of correct refolding under the acidic conditions is also found in the disulfide-reduced form of ovalbumin (16).

Conformational Characteristics of the Initial Burst Intermediate and the Implications for the Serpin Folding—The use of D[367–382]CM-73 described here as the starting denatured state for the refolding analysis provided a direct approach to analyze the structural and functional implications of the IA state for the correct refolding of ovalbumin; D[367–382]CM-73 can refold after rapid dilution into the folding buffer to an initial burst intermediate IA that displays almost exactly the same intrinsic tryptophan fluorescence spectrum as the initial burst intermediates produced from D[367–382] and D[SH] (Fig. 3). Due to the absence of a cysteine counterpart of the native disulfide, the IA intermediate produced from D[367–382]CM-73 should not be transformed into the correct disulfide intermediate IN[73–120] and hence should lack the correct folding ability into the native form. This was indeed the case; no alteration was essentially observed during the refolding time up to 20 h for the intrinsic tryptophan fluorescence spectrum of the IA state produced from D[367–382]CM-73 (Figs. 3 and 4). The stable nature of this IA state enabled us to analyze the conformational state by a variety of probes. The intermediate IA displayed partial regaining (about 58% of the α-helix content) of the native far-UV CD spectrum (Fig. 3), an accessible nature against trypsin (Fig. 4), some ANS binding capacity (Figs. 3 and 4), and flexibility detected by the occurrence of the intrachain sulfhydryl/disulfide exchange (Fig. 6). These characteristics were consistent with a non-native conformational state for the IA state. Nevertheless, the intermediate IA was a compact molecule, similar to the native form, as evaluated by size-exclusion chromatography (Table I).

Alternative stable intermediate states IA produced from D[73–120] and D[SH] have been shown to display a common far-UV CD spectrum to the IA states from the same urea-denatured ovalbumin forms (16). In the present study, the same IA state was confirmed to be produced by dilution of D[367–382] and D[367–382]CM-73 with the acidic buffer (Fig. 3). Likewise, the IA state produced from D[367–382]CM-73

![Diagram of the refolding process of ovalbumin](attachment://image.png)
displayed the same far-UV CD spectrum (Fig. 3). This indicates that the secondary structure contents in the IN and IA states are independent of the differences in the disulfide pairings and pH conditions. The secondary structure may be, therefore, formed in the initial burst intermediate, depending on the helix-forming propensity of local peptide segments, without the help of non-local native interactions. Indeed, the regaining of the native far-UV CD ellipticity in the IN state (58% of the CD ellipticity at 222 nm) is very similar to the percentage (55%) of the value, predicted on the basis of helix-forming propensities of individual amino acids (30), relative to the α-helix content in the crystal structure (11). The IA intermediate, however, cannot refold directly into the native-like conformer NA under the acidic conditions. This may be closely related to the findings that the IA state is a molecular state with a more extended Stokes radius and more exposed hydrophobic core than the IN intermediate (Table 1 and Fig. 3). These conformational properties of IA are similar to that of an intermediate called the “pre-molten globule state,” which has partially folded secondary structures and somehow extended conformation with exposed hydrophobic core and disordered long-range interactions (31).

The native refolding after the initial burst phase should include the reorganization of the preformed secondary structure by which the formations of all the non-local, native interactions are attained. The main body of a serpin comprises the α-helix and β-structure domains. Unlike usual multidomain proteins, the serpin domains are closely packed with each other, and many of the α-helix and β-strand elements reside almost alternately on the primary structure. This unique domain structure might be related to the metastable nature of native serpin. That the intermediate IA with extended conformation cannot undergo the correct folding into the NA state strongly suggests that the partially formed secondary structure elements in the initial burst phase must be reorganized with strong mutual interactions in a compact conformational state of IA that enables subsequent structural reorganization into the metastable native state.

Comparison with the Refolding Mechanism of Other Proteins—The previous experimental results for the native refolding of small single-domain proteins have been consistent with the presence of a specific pathway, such as the two- or multistate transition pathways (32–34), although theoretical analyses have proposed a new mechanism that includes protein folding through undefined multiple pathways within a funnel-like energy landscape (35, 36). Unlike the extensive experimental evidence for small single-domain proteins, the folding mechanism has been poorly understood for large complex proteins. This is due, at least in part, to the difficulty for application of powerful methods for protein folding analysis, such as nuclear magnetic resonance.

As a general approach for a variety size of proteins, the oxidative refolding systems of disulfide proteins have been widely employed. The refolding processes of disulfide proteins may be categorized into three classes depending on the conformational state of disulfide-reduced forms. As first class proteins, most small single-domain proteins, such as bovine pancreatic trypsin inhibitor (37), lysozyme (38), and ribonuclease A (39), essentially assume unfolded conformation in the disulfide-reduced state. In the most deeply investigated example of bovine pancreatic trypsin inhibitor, the refolding proceeds via a well-defined pathway in which partially disulfide-bonded species with unique conformation are included as folding intermediates (37, 40, 41). A serious problem has, however, been pointed out for this oxidative refolding system. The first step for disulfide regeneration is the intermolecular attack of an oxidizing disulfide agent onto protein sulfhydryls; the differential accessibility of the protein sulfhydryls, due to the conformational situations of refolding intermediates, may perturb the true kinetic pathway of the refolding (22, 23).

The second class of disulfide proteins, such as the constant fragment of immunoglobulin light chain (22) and ribonuclease T1, in the presence of a high salt concentration (42), assumes a native-like conformation on their disulfide-reduced state. The third class of the proteins includes ovotransferrin (43–45) and serum albumin (46), which assume partially folded conformations in the disulfide-reduced forms. The urea-denatured forms of these two classes of proteins refold into the fully or partially folded conformation under disulfide-reduced conditions. Subsequent regeneration of the correct intrachain disulfide(s) stabilizes or improves the pre-formed conformations. It is therefore difficult to obtain information about the overall refolding pathway directly from the disulfide regeneration processes of the second and third classes of disulfide proteins.

In the present study, ovalbumin, which is categorized into the second class of disulfide proteins, was analyzed by a differential way. The refolding process involved regaining the native disulfide Cys73-Cys100 from a non-native pairing of D[387–382] by intrachain sulfhydryl/disulfide exchange reactions without the help of any added chemical oxidant. The obtained refolding mechanism includes multiple intermediates that undergo extensive interconversion (Fig. 7). Such a refolding process is considered to be more accurately related to a funnel-like model than a pathway model with a small number of defined intermediates, although the quantitative description by an energy landscape cannot be done at present. The use of D[387–382]CM-73 as the starting denatured state enabled us to analyze the structural characteristics of the initial burst intermediate as a stable form. The obtained results highlighted the unique structural features of IN that are characterized by a compact conformation and a partial regain of the α-helix content that is probably formed by the propensity of a local primary structure. These structural characteristics of IN are very similar to the partially folded intermediate of β-lactoglobulin, which is predominantly β-sheet protein. A refolding intermediate and equilibrium molten globule state of this protein assume a very compact conformation (47, 48), and the polypeptide segment with a good helix propensity takes a non-native helix in an early refolding intermediate (49, 50).

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