Refolding Process of Ovalbumin from Urea-denatured State

EVIDENCE FOR THE INVOLVEMENT OF NONPRODUCTIVE SIDE CHAIN INTERACTIONS IN AN EARLY INTERMEDIATE

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Ovalbumin contains one cystine disulfide (Cys73-Cys120) and four cysteine sulfhydryls (Cys11, Cys30, Cys367, and Cys382) in a single polypeptide chain of 385 amino acid residues. The refolding mechanism of ovalbumin was investigated under disulfide-bonded and disulfide-reduced conditions using the denatured protein state, Dₐ, as the starting protein sample. For the preparation of Dₐ, the disulfide-intact and disulfide-reduced forms of ovalbumin were denatured by protein incubation in 9 M urea at pH 2.2. When Dₐ was placed in a refolding buffer, pH 8.2, an intermediate state Iₐ was produced in either the disulfide-bonded or the disulfide-reduced condition; Iₐ showed about 60% of the native CD ellipticity at 222 nm and the intrinsic tryptophan fluorescence with the native spectrum peak but with decreased intensity. The formation of Iₐ as detected by far UV CD ellipticity was quite rapid and finished within a mixing dead time of 20 ms. When Dₐ was diluted with an acidic buffer, pH 2.2, a partially folded equilibrium intermediate Iₑ with the structural characteristics equivalent to those of Iₐ was formed. After the formations of Iₑ and Iₐ, the regains in CD ellipticity and tryptophan fluorescence at pH 8.2 followed biphasic kinetics in the disulfide-bonded condition but monophasic kinetics in the disulfide-reduced condition. As unexpected findings, the native disulfide in Dₐ and Iₐ underwent nonproductive disulfide rearrangements in the disulfide-bonded condition at an early refolding stage and then was recovered during the subsequent refolding. The integrity of overall refolding was confirmed by the observation that the proteins refolded for 20 h in the disulfide-bonded and disulfide-reduced conditions showed, on differential scanning calorimetry analyses, almost exactly the same denaturation temperatures as their native protein counterparts. These results were consistent with a refolding process for ovalbumin which includes nonproductive side chain-side chain interactions in the early intermediate Iₑ, which requires subsequent reorganization for the correct refolding.

A disulfide protein is a useful model for the investigation of protein folding mechanisms (1). Disulfide proteins that assume fully unfolded (2–11) or partially unfolded conformation (12–14) in their disulfide-reduced states can be oxidatively refolded with the aid of a chemical oxidant. The kinetic folding pathways of some small single-domain proteins, such as bovine pancreatic trypsin inhibitor (2–6), ribonuclease A (7), ribonuclease T1 (8), a-lactalbumin (9), and hirudin (10, 11) have been elucidated by identifying the disulfide structures in partially disulfide-bonded intermediates. A second class of disulfide proteins that assume a native-like conformation in the disulfide-reduced state has also been the target of protein folding studies. The influence of protein conformation on disulfide bond formation in the constant fragment of immunoglobulin light chain (15) and conversely, the influence of disulfide bonds in the folding kinetics of growth hormone (16), have been demonstrated. Ribonuclease T1 that is unfolded by reduction of the two native disulfides assumes a native-like conformation in the presence of a high concentration of salt (17, 18); in recent studies of a one-disulfide mutant of this protein, the mechanism for coupling between the kinetics of sulfhydryl reoxidation and the conformational transitions has been clearly elucidated (19).

As an alternative model for the second class of disulfide proteins, ovalbumin has unique structural characteristics. This egg white protein contains four cysteine sulfhydryls (Cys11, Cys30, Cys367, and Cys382) along with an intrachain disulfide (Cys73-Cys120) in a single polypeptide chain of 385 amino acid residues (20, 21). Our previous studies have shown that the conformational state of the disulfide-reduced ovalbumin is almost indistinguishable from that of the disulfide-bonded form (22). Furthermore, the egg white protein can refold from urea-denatured state in the SH condition, indicating spontaneous protein folding without the native disulfide bond (23). When disulfide-bonded ovalbumin denatured in a high concentration of urea is placed in non-denaturing conditions, the protein refolds much less efficiently than the disulfide-reduced form does (23). This may be related to the observation that many non-native disulfide isomers that have one disulfide and four sulfhydryls in a molecule are produced in a high concentration of urea (24). The detailed refolding mechanism, however, remains to be investigated for both disulfide-bonded and disulfide-reduced ovalbumin.

In the present study we investigated the refolding mechanism of ovalbumin in SS and SH conditions using the denatured protein state, Dₐ, as the starting protein sample. Dₐ was produced by incubation of the disulfide-bonded and disulfide-reduced ovalbumin in 9 M urea at pH 2.2, where possible...
sulfhydryl/disulfide exchange reactions in the disulfide-bonded protein are almost completely blocked (3). Refolding was initiated by a pH jump procedure in which the denatured protein D_{o} was placed in a refolding buffer with near neutral pH. We report here that a partially folded intermediate state, {I}_{N}, is formed very rapidly in either the SS or SH condition as detected by far UV CD and intrinsic tryptophan fluorescence spectra. After the initial burst phase, the time course for the conformational regain followed biphasic kinetics in the SS condition, but it followed monophasic kinetics in the SH condition. According to the data from a peptide mapping analysis, the native sulfide in D_{o} undergoes nonspecific disulfide rearrangements at an early refolding stage in the SS condition and then is recovered during the subsequent slow refolding. These data along with other findings are consistent with a refolding mechanism for ovalbumin which includes nonproductive side-chain side-chain interactions in the early intermediate {I}_{N}, which requires structural reorganization for subsequent correct refolding.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ovalbumin (D_{o}-ovalbumin, diphosphorylated form) was purchased as a fresh egg white by crystallization in an ammonium sulfate solution and subsequent ion exchange column chromatography as described (25, 26). Diphenyldiacarbonyl chloride-treated trypsin (type XI) and chymotrypsin (type II) were purchased from Sigma. *Achromobacter protecinus* (EC 3.4.21.50) was obtained from Wako Pure Chemical Industries.

**Denaturation and Refolding of Ovalbumin**—For the refolding in the SS condition, denatured ovalbumin D_{o} was prepared by incubating the native, disulfide-intact protein at 1.0 mg/ml at 37°C for 30 min in 0.25 M HCl containing 1.0 mM Na-EDTA and 9 mM urea, pH 2.2. Refolding was initiated at 25°C by 20-fold dilution of D_{o} with buffer A (50 mM Tris-HCl buffer, pH 8.6, 1.0 mM Na-EDTA) giving a final pH value of 8.2. The proteins were allowed to refold at 25°C and were then analyzed by intrinsic tryptophan fluorescence and CD spectrum. The buffers were degassed at reduced pressure and equilibrated under N2 atmosphere prior to the refolding. An equilibrium intermediate I_{N} was produced by 20-fold dilution of D_{o} with 50 mM potassium phosphate buffer, pH 2.2, containing 1.0 mM Na-EDTA. The equilibrium intermediate was placed for refolding in a near neutral pH condition, pH 8.2, by being mixed with 0.02 volume of 3.0 M Trizma (Tris) base.

For the experiments in the SH condition, the native sulfide in ovalbumin was fully reduced at 10 mg/ml by incubation with 15 mM dithiothreitol at 37°C for 2 h in 50 mM Tris-HCl buffer, pH 8.2, containing 1.0 mM Na-EDTA (22). Disulfide-reduced D_{o} was produced by 10-fold dilution of the native, disulfide-reduced protein with 0.25 M HCl containing 1.0 mM Na-EDTA and 9 mM urea, pH 2.2. The refolding from D_{o} to the preparation of I_{N} and the refolding from I_{N} in the SH condition were carried out in the same ways as in the SS condition, except that all of the diluents contained 0.25 mM dithiothreitol.

**Measurement of Intrinsic Tryptophan Fluorescence**—The fluorescence spectrum of ovalbumin was measured with a fluorescence spectrophotometer (Hitachi, model F-3000). The intrinsic tryptophan residues in ovalbumin were excited at 295 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. The time course of fluorescence intensity changes was monitored at 338 nm emission. For the spectrum measurements at an early refolding time, the time course of fluorescence intensity changes was monitored at various emission wavelengths with excitation at 295 nm and data at a refolding time of 10 s were plotted.

**CD Spectrum Measurement**—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•cm²/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 refolding time were plotted as a function of wavelength. For rapid mixing experiments, D_{o} was applied to a stopped-flow rapid kinetics access system (Applied Photophysics, RX.1000) attached to the same spectropolarimeter, and changes in CD ellipticity at 230 nm were recorded at 25°C after an 11-fold dilution with buffer A. The mixing dead time that was determined by using 2,6-dichlorophenolindophenol and L-ascorbate (27) was 20 ms.

**Differential Scanning Calorimetry**—D_{o} was refolded for 20 h under the SS and SH conditions, concentrated about 15-fold using a concentrator (Amicon, Centriprep-10), and passed through a Sephadex column (NAP-10, Pharmacia Biotech Inc.) equilibrated with 10 mM sodium phosphate buffer, pH 6.0. Overall recoveries from the original D_{o} forms were about 70%. The refolded proteins and corresponding native protein controls were analyzed by a differential scanning calorimeter (Micro Cal, MCDC-DSC). The protein concentration was 0.4 mg/ml in 10 mM sodium phosphate buffer, pH 6.0. The temperature was scanned at 1 K/min⁻¹.

**Analyses for Disulfide-involved Half-cystines**—At various refolding times in the SS condition, sulfhydryl/disulfide exchanges were quenched by mixing the protein samples with 0.24 volume of 2 M HCl. Disulfide-involved half-cystines were determined by alkylation with a fluorescent reagent, N-iodoacetyl-N'-(5-sulfo-1-naphthyl)ethylendiamine and subsequent peptide mapping procedure as described elsewhere (24).

**RESULTS**

**Folding Intermediate Detected by Optical Methods**—Ovalbumin contains three tryptophan residues, Trp¹⁴⁶ in helix F, Trp¹⁸⁴ as the nearest neighbor residue of the COOH terminus of strand 3A, and Trp²⁶⁰ in helix H (28). The conformational states of different forms of ovalbumin were analyzed by the intrinsic tryptophan fluorescence spectrum. As shown in Fig. 1, the fluorescence emission spectrum of native ovalbumin was indistinguishable for the disulfide-reduced and disulfide-bonded forms; the native proteins showed an emission maximum at 338 nm. D_{o} showed a typical red shift spectrum of an unfolded protein; the emission maximum was shifted to a longer wavelength of 352 nm, and the fluorescence intensity was decreased to 32% of the native form. When D_{o} was refolded at a near neutral pH, the protein showed at the early refolding time of 10 s a fluorescence spectrum that had a peak at the
obtained data, while noisy, were consistent with that. A condition was monitored using a rapid mixing tool, the proteins. When the time course of CD ellipticity changes in the time of 20 h was almost exactly the same as that of the native form, and the CD spectra of the proteins refolded for a prolonged refolding time of 20 h. The absolute ellipticity increased further with time of refolding. The absolute ellipticity at 222 nm and 57% fluorescence intensity at 338 nm of the native form. Figs. 1 and 2 also demonstrate that DA was transformed by 20-fold dilution with potassium phosphate buffer, pH 2.2, into a partially folded form IA. The far UV CD spectrum of IA was almost indistinguishable from that of the early folding intermediate IN. The fluorescence spectrum of IA showed the maximum at the same wavelength (338 nm) as that of IN. The decreased fluorescence intensity for IA can be accounted for by solvent effects by acid, since DA shows decreased fluorescence intensity compared with the urea-denatured ovalbumin at pH 8.2 (Fig. 1). In addition, a previous report has shown that native ovalbumin represents significantly decreased fluorescence (about 55%) at pH 2.2 from that at pH 8.2 but almost indistinguishable far UV CD spectra at the two different pH values (29). The disulfide rearrangements should be almost completely blocked at pH 2.2. Furthermore, the conformational state was constant during 2-h incubation at pH 2.2, as evaluated by the far UV CD analysis (data not shown). We therefore concluded that the acid-quenched IA is an equilibrium intermediate state that possesses structural characteristics equivalent to IN.

Differential Scanning Calorimetry Analysis of Refolded Ovalbumin—The data from the intrinsic fluorescence and far UV CD analyses showed that most if not all ovalbumin molecules refold into the native state at 20-h incubation (Figs. 1 and 2). The integrity of native refolding was investigated more rigorously by an alternative method of differential scanning calorimetry. Fig. 3 demonstrates that the protein refolded in 20 h in the SS condition underwent thermal transition with almost the same melting temperature as did the native protein counterpart; the thermal denaturation temperatures were 77.7°C for the native protein and 77.6°C for the refolded protein. Likewise, the melting temperature for the protein refolded in the SH condition (70.8°C) was almost the same as that for native, disulfide-reduced ovalbumin (70.7°C). The minor peak observed for the refolded protein in the SH condition may be accounted for by reoxidation of the native disulfide during the refolding time of 20 h and/or during the protein concentration procedure prior to the calorimetry analyses, since the peak temperature was about 77°C, which is essentially the same value as the melting temperature for the native, disulfide-bonded protein. The peak area was, however, less than 8% of the major peak. We therefore concluded that most of the ovalbumin molecules can refold correctly in the SS and SH conditions into the same protein energy states as the native proteins.
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Fig. 4. Time course for the refolding after the initial burst phase. Dₐ and Iₐ were prepared as described under “Experimental Procedures.” The time course for the refoldings at pH 8.2, 25 °C from Dₐ (panels A and B) and from Iₐ (panels C and D) in the SS (open circles) and SH (closed circles) conditions were monitored by the intrinsic tryptophan fluorescence at 338 nm (panels A and C) and by CD ellipticity at 222 nm (panels B and D) as described under “Experimental Procedures.” The ordinate shown by F₉(t) represents the fraction of the native form at the refolding time t, which was calculated by the equation F₉(t) = (Xₐ - X₀)(Xₐ - X₀), where X₀ and Xₐ are the values at the refolding times of 6 s and t, respectively. Xₐ is the final value of the refolding; the fluorescence intensity and CD ellipticity at the refolding time of 20 h were employed as X₀. As control experiments, disulfide-bonded and disulfide-reduced ovalbumin preincubated at pH 2.2 in the absence of urea were diluted 20-fold with buffer A giving the same final pH value of 8.2; the CD and fluorescence intensities were confirmed to show invariably the native values during the incubation time of 120 min. The solid curves represent nonlinear least squares fits of the duplicate experimental data to theoretical rate equations (Equations 1 and 2, described under “Discussion”). For the nonlinear least squares fits, a program utilizing a Levenberg-Marquardt algorithm was employed.

Refolding Kinetics after the Initial Burst Phase—The data in Figs. 1 and 2 also show that slow refolding proceeds after rapid formation of the intermediate state. The time courses of the refoldings from Dₐ after the initial burst were examined by intrinsic fluorescence and CD analyses. Fig. 4 (panels A and B) shows the time-dependent increases in the fraction of the refolded protein after 6 s of the refolding from Dₐ. The refolding in the SS condition apparently followed biphasic kinetics (for the quantitative calculation, see “Discussion”). In contrast, ovalbumin refolded with simple monophasic first-order kinetics in the SH condition. No significant difference was observed in either the SS or SH condition for the kinetic profiles obtained by the fluorescence and CD analyses.

The data in Figs. 1 and 2 demonstrate that Dₐ is transformed by dilution with potassium phosphate buffer, pH 2.2, into a partially folded form Iₐ*, which has structural characteristics equivalent to those of Iₐ. To examine whether or not Iₐ* can refold in a manner similar to the refolding from Dₐ, the acid-quenched equilibrium intermediate was placed in a near neutral condition, and the time course of the refolding was monitored by intrinsic fluorescence and CD analyses. As shown in the panels C and D of Fig. 4, Iₐ* refolded with essentially the same kinetics as Dₐ; the refolding followed biphasic kinetics in the SS condition but monophasic kinetics in the SH condition.

Sulfhydryl/Disulfide Exchanges during Refolding—The data in Fig. 4 indicate that the biphasic kinetics are closely related to the presence of the intrachain disulfide bond. The denatured state Dₐ* should contain the native disulfide Cys¹⁷¹, Cys¹²⁰ and four cysteine residues Cys¹¹, Cys³⁰, Cys³⁶⁷, and Cys³⁸² since intrachain sulfhydryl/disulfide exchange reactions possible for fully denatured ovalbumin (24) should be almost completely quenched at the employed condition of pH 2.2 (3). The question, however, arose as to whether the involvement of disulfide rearrangement reactions during the refolding at pH 8.2 results in the more complex kinetics in the SS condition than in the SH condition. To examine the possibility of disulfide rearrangements during the refolding, we determined the disulfide-involved half-cystines by the peptide mapping procedure at various refolding times.

As shown in Fig. 5A, only Cys¹⁷¹ and Cys¹²⁰ were detected as disulfide-involved cysteines at the refolding time 0 from Dₐ*. The disulfide-involved Cys¹⁷¹ and Cys¹²⁰, however, decreased significantly at an early refolding time of 1.0 min; concomitantly at this stage, all of the other four cysteines, Cys¹¹, Cys³⁰, Cys³⁶⁷, and Cys³⁸², were detected as the disulfide-involved cysteines. The amounts as disulfide-involved cysteines were the minima (about 60%) for Cys¹⁷¹ and Cys¹²⁰ but the maxima (about 20%) for the other cysteines at 15 min of the refolding.

The disulfide-involved Cys¹⁷¹ and Cys¹²⁰, however, both increased gradually after 15 min, and the amounts were about 90% at 30-h refolding. The next question was related to the refolding stage that involves the nonproductive disulfide rearrangements. In the refolding system from Dₐ*, ovalbumin denatured under acid-urea conditions was placed in near neutral buffer conditions to be allowed to refold. The original Dₐ*, therefore, may first be transiently transformed by pH jump to the highly denatured state at pH 8.2 and then to the early intermediate state Iₐ by...
conformational conversion. Such a highly denatured state should undergo extensive disulfide rearrangements at near neutral pH (24). However, the data in Figs. 1, 2, and 5A showing that the rapid formation of IN precedes the disulfide rearrangement appeared to support the rearrangement reactions in IN rather than in the transient denatured state. To confirm this, we analyzed disulfide-involved cysteines during the refolding from IA, which has the native disulfide and equivalent structural characteristics to IN, since the involvement of ovalbumin in the transient denatured state can be skipped in this refolding system. The data in Fig. 5B demonstrate that the disulfide-involved Cys73 and Cys120 decreased at an early refolding time in almost the same manner as in the direct refolding from DA. The disulfide-involved Cys73 and Cys120 showed the minima at 15 min and 5 min, respectively, and then both increased gradually; at 20 h of the refolding, the amounts again reached values of about 90%. Almost the same time courses as in the direct refolding from DA were also observed for the other four cysteine residues; the disulfide-involved Cys11, Cys30, Cys367, and Cys382 increased tentatively at an early refolding time and then decreased with the time of the refolding.

**DISCUSSION**

The data in the present report demonstrate that most, if not all, of the ovalbumin molecules in DA refold correctly in either the SS or SH condition into the native form, N, as evaluated by the intrinsic fluorescence and far UV CD spectra (Figs. 1 and 2). Furthermore, the results from the differential scanning calorimetry analysis provide evidence for the equivalent conformational stability of the refolded protein to the native protein counterpart (Fig. 3). To our knowledge, this is the first demonstration that the integrity of native refolding was confirmed by differential scanning calorimetry for such a large protein as ovalbumin. An intermediate state IN is produced from DA at an early stage (within 20 ms) of the refolding at pH 8.2 (Figs. 1 and 2) in either the SS or SH condition. An equivalent equilibrium intermediate IN is formed when DA is diluted with a non-urea acidic buffer (Figs. 1 and 2). As unexpected findings, the native disulfide Cys73-Cys120 undergoes nonproductive disulfide rearrangements due to sulfhydryl/disulfide exchanges with the other four cysteines during the refoldings from DA and IN in the SS condition (Fig. 5). These data are consistent with the following diagram for the refolding process of urea-denatured ovalbumin,

\[
\begin{align*}
\text{IN}^* & \rightarrow \text{IN} \\
\text{IN} & \rightarrow \text{N} \\
\text{IN} & \rightarrow \text{IA}
\end{align*}
\]

where IN represents the mispaired-disulfide intermediate that is formed during the refolding in the SS condition. IN is the intermediate with the native disulfide Cys73-Cys120 (SS condition) or without any intrachain disulfide (SH condition) and assumed to be the competent one for subsequent folding into N with a first-order rate constant, k1. Through the disulfide rearrangements, IN undergoes reversible interconversion with IN, k-1 and k-1 are the first-order rate constants for the conversions from IN to IN and from IN to IN, respectively. The small arrows with thin solid lines represent the initial burst for the conformational transition and/or pH jump steps.

Diagram 1 is consistent with the kinetic data of the refoldings after the initial burst phase (Fig. 4). F(t), which is defined as the fraction of N at a refolding time t, can be expressed in the SS condition as follows.

\[
F_N(t) = 1 + \frac{r_1 + r_2}{r_1 - r_2} \exp(-r_2t) - \frac{r_1 + r_2}{r_1 - r_2} \exp(-r_1t)
\]

(Eq. 1)

where \( r_1 \) and \( r_2 \) are related to the rate constants as \( r_1 = -\alpha + \sqrt{\alpha^2 - \beta} \), \( r_2 = -\alpha - \sqrt{\alpha^2 - \beta} \), \( 2\alpha = k_{-1} + k_{-1} + k_1 \), and \( \beta = k_1k_{-1} \). In the SH condition, no disulfide rearrangements are involved (\( k_{-1} = k_{-1} = 0 \)). Equation 1 can therefore be reduced into a single exponential first-order rate equation.

\[
F_N(t) = 1 - \exp(-k_1t)
\]

(Eq. 2)

As displayed by the solid lines in Fig. 4, the data for the refolding from either DA or IN can be fitted quite adequately to Equation 1 in the SS condition and to Equation 2 in the SH condition. The first-order rate constants obtained by the data fitting analysis are summarized in Table I. The three first-
First, the refolding of ovalbumin follows biphasic kinetics in information about the refolding mechanism of ovalbumin. The refolding data in Fig. 4 to Equation 1 in the SS condition and to Equation 2 in the SH condition.

The fraction of ovalbumin species with the native disulfide can therefore be calculated as a function of t using the rate constants in Table I as defined in the insets of Fig. 5. The values for the disulfide-involved Cys11-Cys30 and Cys120-Cys382, which also includes their participation in mispaired disulfides with the other cysteines, should not be exactly the same as, but should be closely related to, the amount of the protein species with the native disulfide Cys73-Cys120. Fig. 5 represents the theoretical curve is consistent with the experimental data at the qualitative level. The values for FN,SS(t) decrease rapidly at early refolding times showing the minima at 9–10 min and then gradually increasing with the time of the refolding. Such theoretical profiles were very similar to the experimental time courses for the disulfide-involved Cys73-Cys120 and Cys30-Cys382, which show the minima at 5–15 min.

The results from the data-fitting analyses provide important information about the refolding mechanism of ovalbumin. First, the refolding of ovalbumin follows biphasic kinetics in the SS condition (Fig. 4). As a general mechanism for biphasic refolding kinetics, the involvement of the parallel pathway that is related to cis-trans isomerization of proline residues has been demonstrated (30). Ovalbumin indeed contains 14 proline residues. Diagram 1 is, however, consistent with the biphasic kinetics in the SS condition without including parallel pathways. A decelerated folding mechanism in disulfide-bonded ribonuclease T1 which is accounted for by decreased proline isomerization rate (31) is also unlikely in the refolding of ovalbumin, since the folding rate constant $k_1$ is almost identical in the SS and SH conditions (Table I). It is, therefore, very likely for the refolding kinetics of ovalbumin that the effects of the proline isomerization problem, if any, work in an undifferentiated manner in the SS and SH conditions. Second, according to our previous simulation analysis for the disulfide rearrangements in ovalbumin, the rate constant $k$ for a sulfhydryl/disulfide exchange in 9 M urea at pH 8.2 is related to the equation $k = 820 n_e^{-2}$, where $n_e$ is the “effective number” of amino acids separating a disulfide and a relevant sulfhydryl (24). For the initial disulfide rearrangements by the nucleophilic attacks of the four cysteines on the native disulfide Cys73-Cys120, the rate constants should be in the order Cys30 > Cys11 > Cys367 > Cys382, the absolute rates for the productions should be quite variable from the most rapid reaction (attack by Cys30 forming Cys830-Cys73 and Cys30-Cys120) of 0.44 min$^{-1}$ to the slowest reaction (attack by Cys382 forming Cys73-Cys382 and Cys120-Cys382) of 0.012 min$^{-1}$. As shown in Table I, the rate constant for the formation of mispaired disulfides in IN ($k_1$) is approximately half of that for the most rapid reaction in the highly denatured state. Although the value for $k_1$ is an average value for many rearrangement reactions, the data in Fig. 5 represent almost equivalent disulfide participation for Cys11, Cys30, and Cys382, except for a slightly lower value for Cys367. These data may reflect some compact but highly flexible conformational state for IN in which the effective concentrations of the four cysteine sulfhydryls relative to the native disulfide are all significantly high.

The present refolding mechanism of ovalbumin has implications for the current view of the nature of protein folding intermediates. The equilibrium molten globule state has been observed with several globular proteins as a partially denatured form; the state has been claimed to be a general productive intermediate in protein folding (32–34). There is, however, examples in which protein folding proceeds via two-state behavior without an apparent folding intermediate (35, 36). Theoretical approaches for protein folding have suggested that folding intermediates accumulate because of kinetic traps caused by partial misfolding (37, 38). Indeed, experiments with cytochrome c represent that the refolding proceeds via an essentially two-state behavior without any apparent intermediate and that a trapped intermediate under three-state conditions results from misfolding of the polypeptide chain in the initial collapse step (39). In the ovalbumin refolding, IN is formed as an initial burst, and the subsequent regain of the native conformation proceeds slowly (Figs. 1, 2, and 4). The observations that both the native disulfide species $D_A$ and $I_A$ undergo non-native disulfide rearrangements in an early stage of the refolding (Fig. 5) clearly demonstrate that nonspecific side-chain-side chain interactions are involved in an early intermediate of ovalbumin refolding. The early intermediate $I_N$ is therefore very likely to be a molecular collapse intermediate that requires subsequent structural reorganization for the correct refolding.

The data in Fig. 5 represent that the protein species with a mispaired disulfide can refold correctly into the native disulfide form via disulfide rearrangements. A sulfhydryl/disulfide protein that contains, in its natural or engineered form, both cystine and cysteine residues in the native molecule as in ovalbumin may generally work as a useful model for the investigation of a protein folding mechanism. The folding mechanisms of disulfide proteins have been investigated using mostly oxidative refolding systems in which the disulfide-reduced, denatured state is transformed into the native, disulfide-bonded form with the help of an oxidizing agent. A protein disulfide formation, however, includes multiple chemical steps: the first step is the intermolecular attack of a protein sulfhydryl on an oxidizing disulfide agent generating a protein mixed disulfide, and the second step is the intramolecular attack of a second protein sulfhydryl on the mixed disulfide (1). Intrachain sulfhydryl/disulfide exchanges also occur during the refolding of most of the small single-domain proteins (2–11). A major problem encountered in the kinetic analysis for an oxidative refolding pathway is related to the protein sulfhydryl accessibility in the first step to an oxidizing agent, as demonstrated for bovine.

### Refolding Process of Ovalbumin

[Table I](#)

| Refolding from Conditions | Conformational probe$^a$ | Rate constants $k_1$, $k_2$, $k_1$ | min$^{-1}$ |
|--------------------------|------------------------|-----------------------------------|------------|
| $D_A$ SS FL              |                        | 0.011 0.23 0.27                   |            |
| $I_A$ SS FL              |                        | 0.010 0.19 0.23                   |            |
| $D_A$ SH FL              |                        | 0.25                              |            |
| $I_A$ SH FL              |                        | 0.24                              |            |
| $D_A$ SS CD             |                        | 0.0094 0.23 0.21                  |            |
| $I_A$ SS CD             |                        | 0.0092 0.28 0.24                  |            |
| $D_A$ SH CD             |                        | 0.21                              |            |
| $I_A$ SH CD             |                        | 0.16                              |            |

$^a$ FL and CD represent the fluorescence and CD spectrum analyses, respectively.
pancreatic trypsin inhibitor (6). In the refolding process of the sulfhydryl/disulfide protein, however, only intrachain sulfhydryl/disulfide exchange reactions should be included. The sulfhydryl accessibility problem in the first reaction can therefore be circumvented.

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