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Refolding of Urea-Denatured Ovalbumin That Comprises Non-Native Disulfide Isomers

Maki Onda, Eizo Tatsumi,[1] Nobuyuki Takahashi, and Masaaki Hirose[2]

The Research Institute for Food Science, Kyoto University, Uji, Kyoto 611

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Ovalbumin, which contains one cystine disulfide (Cys73-Cys120) and four cysteine sulfhydryls (Cys11, Cys30, Cys367, and Cys382) in the native state, undergoes intrachain sulfhydryl-disulfide exchanges at high concentrations of urea, generating many non-native disulfide isomers [E. Tatsumi, N. Takahashi, and M. Hirose (1994) *J. Biol. Chem.* 269, 28062-28067]. The refolding of ovalbumin from the urea-denatured state was investigated. When the denatured protein was diluted 20-fold with a refolding buffer (pH 8.2), an initial burst intermediate I_N was produced within the 20 ms instrumental dead time; I_N showed about 60% of the native CD ellipticity at 222 nm. The intrinsic tryptophan fluorescence of I_N showed the same peak (338 nm), but with decreased intensity (57%), as compared to the native protein. After the rapid formation of I_N, most of the ovalbumin molecules correctly refolded into the native state with slow biphasic kinetics, as evaluated by far-UV CD, tryptophan fluorescence, and trypsin-resistance analyses. Furthermore, a peptide-mapping analysis revealed that sulfhydryl/disulfide exchange reactions occurred during the refolding, thereby increasing the formation of the native disulfide. The integrity of overall refolding was confirmed by a differential scanning calorimetry analysis. These data were consistent with the view that most, if not all, of the mispaired disulfide isomers in the urea-denatured ovalbumin can correctly refold into the native state *via* intrachain disulfide rearrangements.

Key words: disulfide rearrangement, folding intermediate, ovalbumin, protein folding, refolding.

In vitro, small single-domain proteins (*1-7*) as well as larger multi-domain proteins (*8-11*) can oxidatively refold from their disulfide-reduced, denatured state into the native conformation with the aid of an oxidizing reagent. Oxidative refolding systems of disulfide proteins have been extensively utilized for the investigation of protein folding mechanisms, since major disulfide intermediates involved in the folding pathway can be trapped in stable forms (*1-7*). Protein disulfide regeneration, 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 (*12*). Intrachain sulfhydryl/disulfide exchanges are also involved during the refolding of most of the small single-domain proteins (*1-7*). A major problem encountered in the kinetic analysis of an oxidative refolding pathway is related to protein sulfhydryl accessibility to an oxidizing agent in the first step. In bovine pancreatic trypsin inhibitor, the most extensively characterized example, only the native two-disulfide intermediate [5-55; 30-51] that is produced from other native two-disulfide intermediates by disulfide rearrangements is the productive species for the acquisition of the third native disulfide bond (*1-5*). This is closely related to the inaccessible nature of cysteine sulfhydryls in the non-productive disulfide intermediates (*5*).

As an alternative model disulfide protein, ovalbumin has unique structural characteristics. The egg-white protein contains six cysteine residues (Cys11, Cys30, Cys73, Cys120, Cys367, and Cys382) in a single polypeptide chain of 385 amino acid residues (*13, 14*). As shown in Fig. 1 (*15*), Cys73 and Cys120 form an intrachain disulfide in the native state. Our previous studies have shown that the conformational state of the disulfide-reduced ovalbumin is almost indistinguishable from that of the disulfide-bonded form (*16*). Furthermore, the egg-white protein can correctly refold from the urea-denatured state under disulfide-reduced conditions, indicating the occurrence of spontaneous protein folding without the native disulfide bond (*17*). When the disulfide-bonded ovalbumin that is denatured in 9 M urea at pH 2.2 is refolded at near neutral pH, the native disulfide in the acid-denatured protein undergoes nonspecific disulfide rearrangements in an initial burst intermediate and then is recovered during the subsequent slow refolding (*18*). Essentially the same intermediate is formed during the refolding of the disulfide-reduced protein (*18*). These data along with other evidence are consistent with the following scheme for the refolding process of urea-denatured ovalbumin:

[1] Supported by JSPS Fellowships for Japanese Junior Scientists.
[2] To whom correspondence should be addressed. Phone: +81-774-38-3734, Fax: +81-774-38-3735
Abbreviation: IAEDANS, *N*-iodoacetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine.

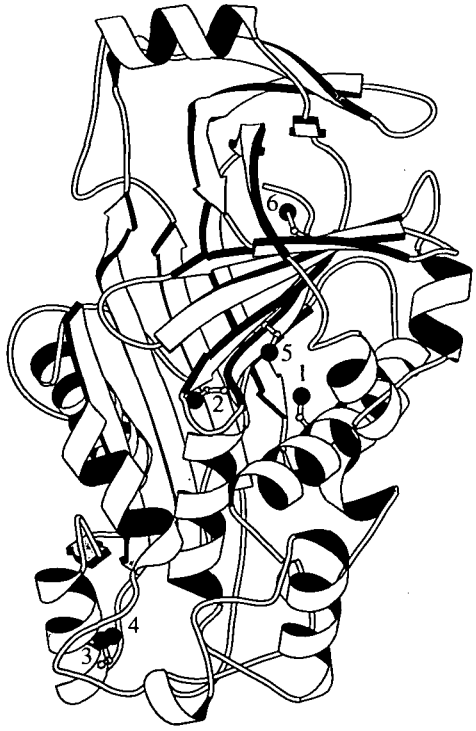

Fig. 1. **Schematic view of ovalbumin.** The figure is based on the X-ray crystallographic data of ovalbumin (*15*) and was drawn using the MolScript program (*29*). The numbered shaded spheres represent the sulfur atoms: 1, Cys11; 2, Cys30; 5, Cys367; 6, Cys382. The sulfur atoms 3 (Cys73) and 4 (Cys120) form the native disulfide bond.

$$\begin{array}{ccccc} & & I_N^* & & \\ & & \uparrow\downarrow & & \\ D & \longrightarrow & I_N & \longrightarrow & N \end{array} \qquad \text{(Scheme 1)}$$

where D is the urea-denatured ovalbumin with the native disulfide Cys73-Cys120. I_N is the initial burst intermediate with the native disulfide or without any intrachain disulfide and is the competent intermediate for subsequent folding into the native form N with or without the native disulfide. I_N^* is the mispaired-disulfide intermediate that is formed during the refolding in the disulfide-bonded ovalbumin; through intrachain disulfide rearrangement reactions, I_N^* undergoes reversible interconversion with I_N.

In the present study, we investigated whether or not the urea-denatured ovalbumin comprising non-native disulfide isomers, D*, can refold into N with the correct disulfide pairing. As demonstrated in a previous report (*19*), many non-native disulfide isomers that have one disulfide and four sulfhydryls in a molecule are produced, when disulfide-bonded ovalbumin is denatured at high concentrations of urea at near neutral pH. According to the X-ray crystallographic data (*15*), however, only the native disulfide Cys73-Cys120 can adopt the native conformation (Fig. 1). The refolding process of ovalbumin denatured in 9 M urea at pH 8.2 was investigated by using several different structural approaches. We report here that most, if not all, of the D* species can correctly refold into the native form *via* disulfide rearrangements according to an extended version of Scheme 1.

EXPERIMENTAL PROCEDURES

Materials—A_1-ovalbumin (diphosphorylated form) was purified by crystallization in an ammonium sulfate solution (*20*) and by subsequent ion-exchange chromatography as described (*21*). Diphenylcarbamyl chloride-treated trypsin (Type XI) and chymotrypsin (Type II) were purchased from Sigma. *Achromobacter* protease I [EC 3.4.21.50] was obtained from Wako Pure Chemical Industries.

Denaturation and Refolding of Ovalbumin—Denatured ovalbumin was prepared by incubating the native, disulfide-bonded protein at 1.0 mg/ml, 37°C for 30 min in Buffer A (50 mM Tris-HCl buffer, pH 8.2, 1.0 mM Na-EDTA) containing 9 M urea. Refolding was initiated at 25°C by 20-fold dilution of the denatured protein with Buffer A, giving a final urea concentration as low as 0.45 M. The proteins were allowed to refold at the same temperature, and then analyzed for trypsin resistance, intrinsic tryptophan fluorescence and CD spectrum. An acid-quenched equilibrium intermediate was produced by 20-fold dilution of the urea-denatured protein with 50 mM K-phosphate buffer, pH 2.2, containing 1.0 mM Na-EDTA. The buffers were degassed at reduced pressure and equilibrated under an N_2 atmosphere prior to the refolding experiments.

CD Spectrum Measurement—The far-UV CD spectrum of ovalbumin was recorded with a spectropolarimeter (JASCO, J-720). The temperature of the cuvette was maintained at 25°C with a circulating water bath. The CD data were expressed as mean residue ellipticity (degree·m^2/decimol) by using a value of 111 as the mean residue weight of ovalbumin.

CD spectra at a short refolding time were determined by measuring the time-dependent increase in absolute CD ellipticity at various wavelengths and the values at a refolding time of 10 s were plotted as a function of wavelength.

For rapid mixing experiments, a stopped-flow rapid kinetics accessory (Applied Photophysics, RX.1000) attached to the same spectropolarimeter was employed and changes in the CD ellipticity of the urea-denatured ovalbumin at 230 nm were recorded at 25°C after 10-fold dilution with Buffer A. The dead time for mixing, determined by using the reaction between 2,6-dichlorophenolindophenol and L-ascorbate (*22*), was 20 ms.

Measurement of Intrinsic Tryptophan Fluorescence—The fluorescence spectrum of ovalbumin was measured with a Hitachi fluorescence spectrophotometer (Model F-3000). The intrinsic tryptophan residues in ovalbumin were excited at 295 nm, and the 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 change was monitored at 338 nm emission. For the spectral 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.

Trypsin-Resistance Assay—Refolded ovalbumin with the native conformation is considered to be a trypsin-resistant species as described (*17*). At various refolding times, the protein was mixed with 0.01 volume of different concentrations (1.25 or 12.5 mg/ml) of trypsin and digest-

ed at 25°C for 1 min. The digestion was terminated by addition of soybean trypsin inhibitor. The proteins were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel (10% polyacrylamide/0.27% bisacrylamide) according to the standard method of Laemmli (*23*), and then stained with Coomassie Brilliant Blue R-250. The amount of trypsin-resistant ovalbumin was determined from the band intensity, which was measured with a densitometer (Shimadzu, CS-9000).

Analyses for Disulfide-Involved Half-Cystines—At various refolding times, 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 IAEDANS and a subsequent peptide mapping procedure as described (*19*). Briefly, the acid-quenched protein was neutralized and alkylated with 0.1 M iodoacetamide in 9 M urea solution. The alkylated protein was precipitated in a cold acetone-HCl solution, dissolved in a urea solution, reduced with dithiothreitol, and then modified with a fluorescent reagent, IAEDANS. The modified protein was extensively proteolyzed with the combination of trypsin, chymotrypsin, and *Achromobacter* protease I. The resultant peptides were analyzed by reversed-phase high-performance liquid chromatography with fluorescence monitoring (excitation, 340 nm; emission, 520 nm). For the standard experiment, the intact ovalbumin was fully reduced with dithiothreitol, all cysteine residues were labeled with IAEDANS, and the molecule was proteolyzed in the same way.

Determination of Cysteine Sulfhydryls by Amino Acid Analysis—The quenching of sulfhydryl-disulfide exchanges in the refolding protein by acid, alkylation of free cysteine sulfhydryls with iodoacetamide, and protein precipitation in a cold acetone-HCl solution were carried out in the same way as above. The alkylated protein was hydrolyzed in 6 N HCl containing 0.1% (v/v) phenol in the gas phase at 110°C for 20 h under vacuum. The carboxymethyl cysteine was determined with an amino acid analyzer (Hitachi, L-8500A).

Differential Scanning Calorimetry—The denatured protein was refolded for 20 h, concentrated about 15-fold using a concentrator (Amicon, Centriprep-10), and passed through a Sephadex column (Pharmacia Biotech, NAP-10) equilibrated with 10 mM Na-phosphate buffer, pH 6.0. Overall recoveries from the original denatured protein were about 70%. The refolded protein and native protein control were analyzed with a differential scanning calorimeter (Micro Cal, MCS-DSC). The protein concentration was 0.4 mg/ml in 10 mM Na-phosphate buffer, pH 6.0. The rate of temperature change was 1 $K \cdot min^{-1}$.

RESULTS

Folding Intermediate Detected by Far-UV CD and Intrinsic Tryptophan Fluorescence Spectra—In a previous study (*18*), the refolding mechanism of ovalbumin in either a disulfide-bonded or a disulfide-reduced condition was investigated using a starting denatured protein sample produced by protein incubation in 9 M urea at pH 2.2, where possible sulfhydryl/disulfide exchange reactions are almost completely blocked; the refolding was initiated by a pH-jump procedure in which the denatured protein is placed in a refolding buffer of near-neutral pH. A partially folded intermediate state, I_N, has been shown to be formed in an initial burst phase, as detected by far-UV CD and intrinsic tryptophan fluorescence spectra, in either the disulfide-bonded or disulfide-reduced condition. The partially folded equilibrium intermediate with structural characteristics equivalent to those of I_N is formed upon the dilution of the acid/urea-denatured ovalbumin with an acidic buffer, pH 2.2.

In the present study, we examined similarly whether or not the initial burst intermediate is also formed during the refolding from the urea-denatured ovalbumin at near neutral pH, where different disulfide isomers should be present (*19*). As shown in Fig. 2, the refolding proteins from the urea-denatured state showed at the early stage of 10 s an intermediate CD spectrum that had 60% of the absolute ellipticity at 222 nm of the native form. When the time course of CD ellipticity change was monitored using a rapid mixing apparatus, the obtained data were consistent with completion of the formation of the intermediate from the urea-denatured state within the mixing dead time of 20 ms (Fig. 2, inset).

Ovalbumin contains three tryptophan residues, W148 in helix F, W184 as the nearest neighbor residue of the C-terminus of strand 3A and W267 in helix H (*15*). As shown in Fig. 3, the fluorescence emission spectrum of the

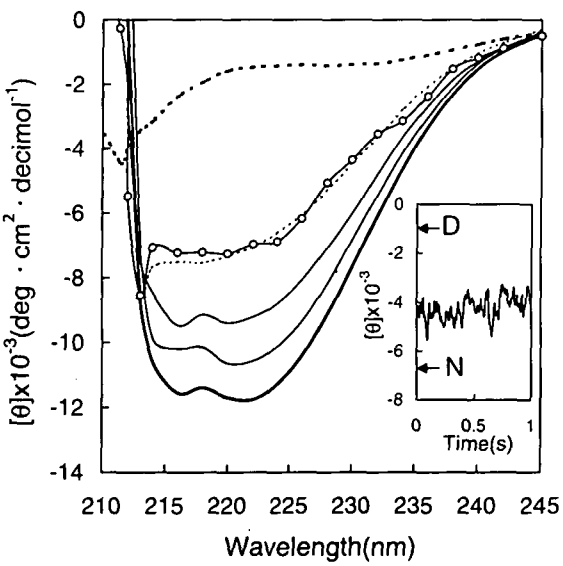

Fig. 2. **Far-UV CD spectra of various states of ovalbumin.** The far-UV CD spectra of native ovalbumin (thick solid line), the protein denatured in 9 M urea (thick broken line), and the proteins refolded for 30 min and 20 h (the upper and lower thin solid lines, respectively) were recorded at 25°C as described in the text. The equilibrium intermediate state was produced by 20-fold dilution of the urea-denatured protein with 50 mM K-phosphate buffer, pH 2.2 containing 1.0 mM Na-EDTA and the CD spectrum was recorded in the same way (thin dotted line). For the CD spectra for early refolding intermediates, the time course of changes in CD ellipticities at different wavelength were recorded during the refolding and the values at the refolding time of 10 s were plotted as a function of wavelength (open circles with thin solid line). In the inset, the urea-denatured protein was refolded at pH 8.2 and the changes in CD ellipticities at 230 nm were recorded for 1 s using a stopped-flow instrument as described in the text. D and N shown by arrows represent the CD ellipticities at 230 nm of the urea denatured and native proteins, respectively.

native form had an emission maximum at 338 nm. The urea-denatured protein showed a typical red-shifted spectrum of an unfolded protein: the emission maximum was shifted to a longer wavelength of 352 nm, and the fluorescence intensity was decreased to 56% of the native form. When the urea-denatured ovalbumin was refolded, the protein showed at an early refolding time of 10 s a fluorescence spectrum that had a peak at the same wavelength, but with much decreased intensity as compared with the spectrum of native ovalbumin.

Figures 2 and 3 also show that after the initial burst phase, the absolute CD ellipticity and fluorescence intensity then increased slowly with increasing time of refolding. The protein refolded for a prolonged time of 20 h showed 90% of the absolute CD ellipticity at 222 nm and 83% of the intrinsic fluorescence intensity at 338 nm of the native protein. The data from the two conformational analyses were therefore consistent with the formation of an initial-burst intermediate having 60% of the absolute CD ellipticity at 222 nm and 57% of the fluorescence intensity at 338 nm of the native form, during the refolding process.

The partially folded intermediate showing the same far-UV CD spectrum as the intermediate formed at pH 8.2 was also formed by dilution of the urea-denatured protein with an acidic buffer, pH 2.2 (Fig. 2). Under the same conditions, the fluorescence spectrum of ovalbumin showed the maximum at 338 nm that is consistent with the peak wavelength for the intermediate formed at pH 8.2 (Fig. 3). The decreased fluorescence intensity for the acid-quenched intermediate can be accounted for by solvent effects owing to acid (*18, 24*). These spectral profiles were almost exactly the same as those of the previously observed intermediates formed from the acid/urea-denatured protein (*18*).

Time Course of the Refolding after the Initial Burst Phase—In a previous report (*17*), we have shown that a trypsin-resistance assay is a sensitive probe for the analysis of the native conformation of ovalbumin. The trypsin resistance of different conformational states of ovalbumin was examined in more detail. As shown in Fig. 4, native ovalbumin was resistant to trypsin at protease concentrations up to 125 μg/ml. Almost all of the urea-denatured protein, however, was degraded at a trypsin concentration as low as 12.5 μg/ml. Likewise, the acid-quenched intermediate that had structural characteristics equivalent to the initial burst refolding intermediate was almost completely degraded at the trypsin concentration of 12.5 μg/ml. This protease-sensitive nature should not be accounted for by the pH-jump procedure for the analysis of the acid-quenched intermediate, since native ovalbumin that had been pre-incubated at pH 2.2 showed protease resistance at 125 μg/ml of trypsin. The data in Fig. 4 therefore imply that the native protein, but not the urea-denatured or initial burst intermediate, is detected as the resistant molecule at a protease concentration higher than 12.5 μg/ml.

The time course of the refolding was examined by means of the trypsin resistance assay and the data were compared with the time courses, after the initial burst phase, obtained by the far-UV CD and intrinsic fluorescence anal-

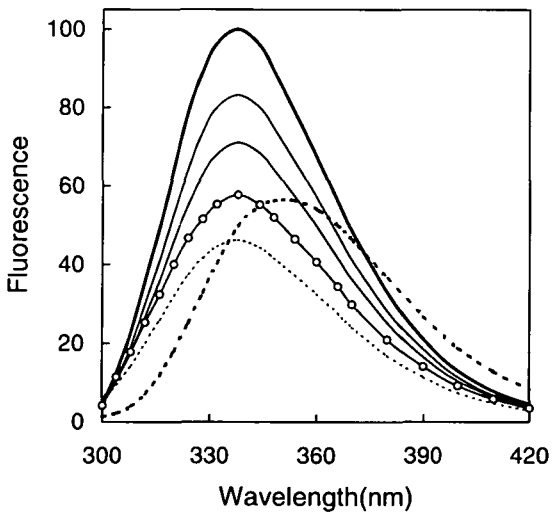

Fig. 3. **Fluorescence emission spectra.** The tryptophan residues in native ovalbumin (thick solid line) and in the protein denatured in 9 M urea (thick broken line) were excited at 295 nm, and the emission spectra were recorded at 25°C. The intrinsic tryptophan fluorescence spectra of the protein refolded for 30 min and 20 h (lower and upper thin solid lines, respectively) were recorded in the same way. The thin dotted line represents the fluorescence spectrum for the equilibrium intermediate state that was produced by 20-fold dilution of the urea-denatured protein with 50 mM K-phosphate buffer, pH 2.2, containing 1.0 mM Na-EDTA. For the fluorescence spectra at an early refolding time, the time course of fluorescence emission changes were recorded during the refolding at different emission wavelengths and the values at refolding time 10 s were plotted as a function of emission wavelength (open circles with thin solid line). The fluorescence intensity is shown in arbitrary units.

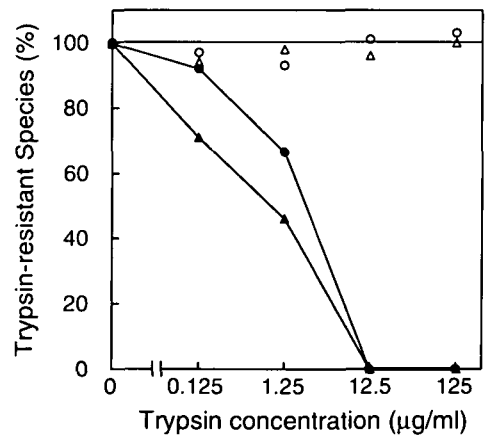

Fig. 4. **Trypsin resistance of various states of ovalbumin.** Native ovalbumin dissolved at 1.0 mg/ml in Buffer A (open circles) or in 50 mM K-phosphate buffer, pH 2.2, containing 1.0 mM Na-EDTA (open triangles), or the protein denatured in Buffer A containing 9 M urea (closed circles) was diluted 20-fold with Buffer A containing different concentrations of trypsin to give the final protease concentrations shown on the abscissa. After 1 min of incubation at 25°C, proteolysis was terminated by addition of soybean trypsin inhibitor. The samples were analyzed for sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the amounts of intact ovalbumin were estimated as described in the text. The equilibrium intermediate (closed triangles) that had been produced by 20-fold dilution of the urea-denatured protein with 50 mM K-phosphate buffer, pH 2.2 containing 1.0 mM Na-EDTA was also analyzed in the same way. The ordinate represents the amounts of the intact proteins at various protease concentrations, expressed as percentages of the values obtained without added protease.

yses. Figure 5 clearly demonstrates that the time course of the refolding was almost exactly the same for the three conformational probes. The refolding data were fitted well to a biphasic rate equation (the sum of two exponentials). Such biphasic kinetics are consistent with the involvement of disulfide rearrangements during the refolding (see "DISCUSSION").

Sulfhydryl-Disulfide Exchanges during Refolding—The preceding data demonstrate the involvement of an initial burst intermediate in the refolding process of the urea-denatured ovalbumin. Our previous study has demonstrated that ovalbumin undergoes, in the initial burst intermediate, disulfide rearrangements *via* intrachain sulfhydryl/disulfide exchange reactions (see Scheme 1). These data suggest that non-native disulfide isomers included in the urea-denatured ovalbumin are refolded into the native form with the correct disulfide bond through disulfide rearrangements.

To investigate this possibility, we determined the disulfide-involved half-cystines by means of the peptide-mapping procedure at various refolding times. As shown in Fig. 6, any of the six cysteine residues was found to be involved in a disulfide bond at the refolding time 0. Such a random distribution in the denatured protein rearranged in a slow reaction to a less random state. For Cys73 and Cys120 that form the native disulfide, the disulfide-involved amounts were only 20% at refolding time 0, but they increased with time of refolding. More than 70% of these cysteines participated in disulfides at a prolonged refolding time of 20 h. In contrast, for the other four cysteines the disulfide-involved amounts decreased with time of refolding. During the refolding, the number of free cysteine sulfhydryls was almost constant; the values were from 3.7 to 3.8, being essentially consistent with the number of free cysteine sulfhydryls in native ovalbumin. These data are consistent with the view that most, if not all, of the denatured

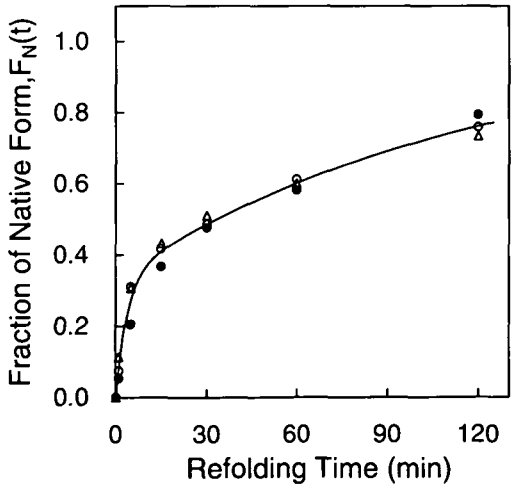

Fig. 5. **Time course of refolding after the initial burst phase.** The urea-denatured protein was refolded at pH 8.2, 25°C, and the time-dependent conformational regain was monitored in terms of the CD ellipticity at 222 nm (closed circles), the intrinsic tryptophan fluorescence at 338 nm (open triangles), and the trypsin resistance (open circles) as described in the text. The ordinate shown by $F_N(t)$ represents the fraction of the native form at the refolding time of t, calculated by using the equation: $F_N(t)=(X_o-X_t)/(X_o-X_N)$, where X_o and X_t are the initial values and the values at the refolding times of t, respectively. For the CD and fluorescence analyses, the values at 6-s refolding were taken as X_o. X_N is the final value of the refolding. The values at the refolding time of 20 h were employed as X_N; the X_N values were 90, 83, and 80% of the native values for the CD ellipticity, tryptophan fluorescence, and trypsin resistance analyses, respectively. The solid curves represent nonlinear least-squares fits of the experimental data to a biphasic rate equation: $F_N(t)=A_1+A_2e^{-k_1t}+A_3e^{-k_2t}$. Obtained constants were: 1.008 for A_1, −0.670 for A_2, −0.340 for A_3, 0.00831 for k_1, and 0.261 for k_2.

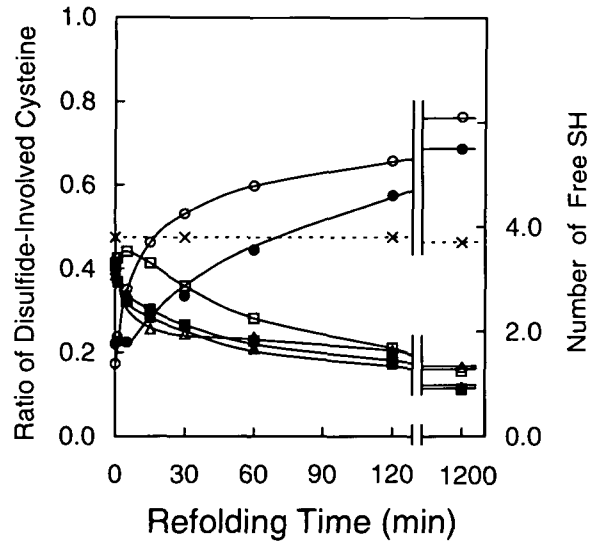

Fig. 6. **Disulfide rearrangements during refolding.** At various times of refolding from the urea-denatured state, disulfide-involved cysteines for Cys11 (open squares), Cys30 (closed squares), Cys73 (closed circles), Cys120 (open circles), Cys367 (closed triangles), and Cys382 (open triangles) were determined using a peptide mapping analysis as described in the text. The number of free cysteine sulfhydryls (cross) was determined by amino acid analysis.

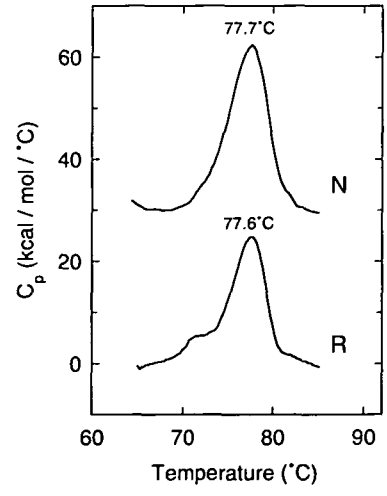

Fig. 7. **Differential scanning calorimetry analysis of the refolded ovalbumin.** Ovalbumin refolded for 20 h and native protein as a control were analyzed by differential scanning calorimetry in 10 mM Na-phosphate buffer, pH 6.0, with a temperature scanning rate of 1 K·min^{-1}. Endothermic transition profiles for the native protein (N) and for the refolded protein (R) are arbitrarily shifted on the ordinate scale for clarity.

non-native disulfide isomers can refold through intrachain sulfhydryl/disulfide exchange reactions into the native disulfide form.

Analysis of the Integrity of the Refolding by Differential Scanning Calorimetry—The preceding data show that most, if not all, of the ovalbumin molecules refold into the native state within 20 h incubation (Figs. 5 and 6). The integrity of the refolding was also investigated by an alternative method of differential scanning calorimetry. As demonstrated in Fig. 7, the protein refolded for 20 h showed a major thermal transition peak at 77.6°C, although a minor peak with lower melting temperature was also detected. The major transition temperature was almost exactly the same as the value for native ovalbumin (77.7°C).

DISCUSSION

In a previous study (*18*), we investigated the refolding mechanism of ovalbumin using the acid/urea-denatured protein (in 9 M urea, pH 2.2) as the starting protein sample, since possible sulfhydryl/disulfide exchange reactions in urea-denatured ovalbumin are almost completely blocked (*3*). Refolding was initiated by a pH-jump procedure in which the acid/urea-denatured protein is placed in a refolding buffer of near-neutral pH (pH 8.2). It has been demonstrated using this refolding system that most of the denatured ovalbumin molecules can correctly refold *via* non-productive disulfide rearrangements in an initial burst intermediate I_N (see Scheme 1 in "Introduction"). In the present study, ovalbumin was denatured at pH 8.2 in 9 M urea as the starting protein sample and then allowed to refold at the same pH value. Since the isoelectric point of A1-ovalbumin is 4.58 (*25*), the electrostatic interactions should be quite different in the urea-denatured conditions at the two different pH values. We have, however, observed that ovalbumin is in a random-coil state in the presence of a high concentration of urea either at pH 8.2 (*19*) or at pH 2.2 (Tatsumi, E. and Hirose, M., unpublished observation). This indicates that both systems allow examination of the refolding process from random-coil state to native state. The major difference in the two refolding systems is in the disulfide structures of the urea-denatured proteins: in a high concentration of urea at a near-neutral pH, but not at a strongly acidic pH, ovalbumin has been shown to undergo extensive disulfide rearrangements generating many disulfide isomers including the native disulfide isomer D and mispaired disulfide isomers D* that all contain one disulfide and four sulfhydryls in the molecule (*19*).

The results of optical and trypsin-resistance analyses in the present report demonstrate that most, if not all, of the urea-denatured D and D* can refold into the native state (Fig. 5). In addition, the differential scanning calorimetry analysis revealed almost exactly the same denaturation temperature for the refolded and native proteins, although a minor shoulder peak with a lower denaturation temperature was detected for the former protein form (Fig. 7). During the refolding, a partially folded intermediate state was formed in the initial burst phase (Figs. 2 and 3); the far-UV CD and intrinsic tryptophan fluorescence spectra were almost exactly the same as those of the early intermediate state formed from the acid/urea-denatured protein (*18*). After the initial burst phase, regain of the native disulfide *via* disulfide rearrangements was observed during the refolding, in which the number of free cysteine sulfhydryls was almost constant (Fig. 6). These data were consistent with refolding of ovalbumin according to an extended version of Scheme 1:

$$\begin{array}{ccccc} D^* & \longrightarrow & I_N^* & & \\ \uparrow\downarrow & & \uparrow\downarrow & & \\ D & \longrightarrow & I_N & \longrightarrow & N \end{array} \qquad \text{(Scheme 2)}$$

where I_N and I_N^* are the initial burst refolding intermediate with the native disulfide and with a mispaired disulfide, respectively; the former is the competent intermediate for subsequent folding into the native form, N. Through disulfide rearrangements, I_N undergoes reversible interconversion with I_N^*, and D with D*.

The data for the conformational regain after the initial burst phase fitted well to an equation consisting of the sum of two exponentials (see the legend of Fig. 5). The apparent rate constants obtained by the data fitting analysis (0.00831 for k_1, and 0.261 for k_2) should include both the first-order rate constants for the disulfide rearrangements and for the folding from I_N to N (*18*). Although, because of the lack for the initial values for D and D*, the rate constants for disulfide rearrangement and folding reactions could not be determined using a more sophisticated rate equation (*18*), the data in Fig. 5 clearly demonstrate that the time course for the conformational regain followed biphasic kinetics. As a mechanism for biphasic refolding kinetics, the involvement of the parallel pathway that is related to *cis-trans* isomerization of proline residues has been demonstrated (*26*). Ovalbumin contains 14 proline residues (*13*). As shown in a previous report (*18*), however, the biphasic refolding kinetics of disulfide-bonded ovalbumin can be accounted for by the involvement of disulfide rearrangements rather than by proline isomerization, since the disulfide-reduced ovalbumin refolds with simple monophasic kinetics.

Our attempts to separate different disulfide isomers by ion-exchange or reversed-phase high-performance liquid chromatography have all been unsuccessful, probably because of the large size of ovalbumin. This has made it difficult to determine in detail the pathway of the disulfide rearrangements during the refolding. Scheme 2, however, indicates that a protein that contains a cystine disulfide along with cysteine sulfhydryls in the molecule may generally be a useful model for the investigation of protein folding mechanisms. First, if an anaerobic condition is employed, the numbers of intrachain disulfide and sulfhydryls are the same as those in the native protein during the refolding in which disulfide rearrangements are included (Fig. 6). This indicates that non-native disulfide isomers can refold into the native disulfide form by intrachain sulfhydryl/disulfide exchange reactions without the help of a catalytic reagent or of an enzyme. A major problem encountered in oxidative refolding studies, related to protein sulfhydryl accessibility to an oxidizing agent (see "Introduction"), can therefore be circumvented in the sulfhydryl/disulfide protein. Second, the unfolded protein that is usually employed as the starting protein for subsequent refolding should consist of a vast number of conformational isomers. The possibility that different subsets of the conformational isomers refold at different rates can not be ruled out. A disulfide isomer that is produced in a sulfhydryl/disulfide protein under denaturing conditions

corresponds to a subset of conformational isomers; the conformational entropy of an isomer depends on the number of amino acid residues separating the two half-cystine residues (*19, 27, 28*). If the refolding mechanisms are compared for different disulfide isomers, the refolding pathway may be directly related to the free energies of the original denatured states.

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