Partially Folded State of the Disulfide-reduced N-terminal Half-molecule of Ovotransferrin as a Renaturation Intermediate*

Masaaki Hirose† and Honami Yamashita

From the Research Institute for Food Science, Kyoto University, Uji, Kyoto 611, Japan

A previous report (Hirose, M., Akuta, T., and Taka-hashi, N. (1989) J. Biol. Chem. 264, 16867-16872) has shown that for the efficient oxidative refolding of disulfide-reduced ovotransferrin, a preincubation under reduced conditions at a low temperature is essential. To study the renaturation pathway, the disulfide-reduced N-terminal half-molecule of ovotransferrin was analyzed by CD spectrum. The reduced protein was found to take, at low temperatures, a partially folded conformation that can be distinguished from both the native and denatured states. The folded protein was in a metastable state with $\Delta G_0$ value of 2.2–2.8 kcal/mol at 6°C. The conformation was variable depending on temperature conditions; its stability was decreased at a lower temperature (1.0–1.2 kcal/mol at 0°C). Subsequent reoxidation at 6°C by oxidized glutathione led efficiently the reduced protein to the correctly renatured form having the iron-binding capacity, indicating that the partially folded state is the immediate precursor to subsequent oxidative refolding.

The mechanism of protein folding is one of the most important subjects in bioscience. The major difficulty in understanding a folding pathway is analyzing the structural properties of intermediate protein species, since they are usually detected as a short-lived state only. A model system that includes the oxidative refolding of a disulfide-reduced protein allows one to investigate the detailed pathway of protein folding (1). The intrachain disulfide is only the natural covalent cross-link that is closely correlated with protein conformation; the formation of intrachain disulfides reflects geometrical vicinity of two relevant sulfhydryls in intermediate species (1, 2). As in the most well documented example of bovine pancreatic trypsin inhibitor, several different intermediate species can be trapped during oxidative refolding, and the folding pathway can be quantitatively elucidated (3–5).

Another possible advantage in investigating a disulfide protein is that the disulfide-reduced state is not short lived, unless the protein is transferred to oxidized conditions. If a disulfide-reduced protein takes a unique form that can be differentiated from native and denatured forms, structural information about an intermediate prior to sulfhydryl reoxidation could be obtained by a conventional method for protein analysis. Data in previous reports, however, appear inconsistent with the occurrence of such a unique state with the fully reduced form of a disulfide protein. The reduced forms of the Fc fragment of immunoglobulin (6) and human growth hormone (7) take almost exactly the same conformation as the disulfide-bonded native forms, as evaluated by far ultraviolet CD spectra. In contrast, bovine pancreatic trypsin inhibitor (8) and RNase A (9) show CD spectra similar to the denatured state in their fully reduced forms. Reduced RNase T₂ that is unfolded under physiological conditions takes native-like conformation in the presence of a high concentration of salt (10) and exerts the enzyme activity in the presence of spermine or at a low temperature (11, 12). The disulfide-reduced form of a single-domain protein, therefore, takes an extreme state, denatured or native-like state:

$$D_{\text{SH}} \leftrightarrow N_{\text{SH}}$$

**Scheme a**

where $D_{\text{SH}}$ and $N_{\text{SH}}$ represent the denatured and native-like state of the fully reduced form of a disulfide protein, respectively. The predominance of either $D_{\text{SH}}$ or $N_{\text{SH}}$ may depend on protein species or on solvent conditions. With regard to the reduced forms of multidomain proteins, little is known about their conformation.

Transferrin is a homologous group of iron-binding proteins that consist of a single polypeptide chain with a molecular mass of about 78,000 Da (13). The N-terminal and C-terminal halves of the chain form independent large domains (N-lobe and C-lobe), each containing one specific iron-binding site (13–15). Each lobe consists of two domains (domain I and II) (14, 15). With regard to ovotransferrin, 15 intrachain disulfides are scattered among all the four domains (16). Our previous report has shown that the fully reduced, denatured form of ovotransferrin can be efficiently renatured by a two-step procedure, which includes an incubation, in the first step, of the fully reduced form at a low temperature and in the second step, reoxidation at a higher temperature in the presence of oxidized glutathione (17). The reduced form in the first step shows a CD spectrum that is an intermediary between those of the native and denatured forms (17). Our question has been that such intermediary spectrum is accounted for by a unique conformation that is differentiated from those of $N_{\text{SH}}$ and $D_{\text{SH}}$ or by an equilibrium mixture consisting of the two extremes. We have investigated differentiating the two possibilities using a less complex protein fragment, the N-terminal half-molecule of ovotransferrin ($M_r$, 36,400, six intrachain disulfides). Here we show that the disulfide-reduced half-molecule takes a unique conformation that is differentiated from $D_{\text{SH}}$ and $N_{\text{SH}}$ and that the state is the immediate precursor to correct oxidative refolding.

**EXPERIMENTAL PROCEDURES**

*Materials—The N-terminal and C-terminal half-molecules of ovotransferrin were prepared as described previously (18). Trypsin (type

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† To whom correspondence should be addressed.
XI, soybean trypsin inhibitor (type I-S), and carboxypeptidase B (type I-DFP) were purchased from Sigma. Carboxypeptidase Y was purchased from Oriental Yeast, Co. GSH and GSSG were obtained from Dojin. Urea and GdnHCl were specifically prepared reagent grade and other chemicals were reagent grade from Nacalai Tesque.

Denaturation and Denatured Reduction of Ovotransferrin—Reduced, denatured proteins were prepared by the incubation of the N-terminal and C-terminal half-molecules with 5 mM DTT at 37 °C for 30 min in buffer A (8 M urea, 1.0 mM NaEDTA, 50 mM Tris-HCl, pH 8.0). Refolding was initiated by a dilution of the reduced, denatured protein with a refolding buffer. All the buffers were degassed at reduced pressure prior to the experiments. The reduced, denatured proteins were freshly prepared for every experiment.

Analyses by CD Spectroscopy—The reduced, denatured protein (12 mg/ml) was diluted 200-fold with buffer B (25 mM Tris-HCl, pH 8.5 at 0 °C) containing 0.5 mM DTT at a low temperature. After an incubation at the same temperature for 5 min, CD spectra were measured at the same temperature or at a shifted temperature with a Jasco J-501C spectropolarimeter, using a 0.2-cm cuvette. The CD spectra of the native half-molecule and of the reduced, denatured protein were determined at 0.12 mg/ml of a protein concentration in buffer B and in buffer B containing 0.5 mM DTT and 4.5 M urea, respectively, using a 0.1-cm cuvette. The temperature of samples, which was directly monitored with a thermistor sensor, was maintained with a circulating water bath. The CD spectra were determined at least three times on different days, and the results were averaged. They were expressed in mean residue ellipticity (degree cm$^2$/dmmol), using the mean residue weights of 110 for the N-terminal half-molecule and 111 for the C-terminal half-molecule.

The protein stability was examined by measuring ellipticity at 222 nm in the presence of various concentrations of GdnHCl. The reduced, denatured protein was diluted 200-fold with buffer B containing 0.5 mM DTT at 0 or 6 °C and preincubated at the same temperature for 5 min. The sample was mixed with 0.6 volume of buffer B containing 0.5 mM DTT and different concentrations of GdnHCl, incubated at the same temperature for 7 min, and then analyzed for molar ellipticity at 222 nm. The stability of the native half-molecule was determined in the same way, except that DTT was excluded from all the buffers. Data for triplicate measurements were averaged.

Reoxidation of the Reduced, Denatured Form—The reduced, denatured N-terminal half-molecule (2.0 mg/ml) was diluted 100-fold at 0 or 6 °C with buffer B containing 1.0 mM GSH and 0.1 mM NaEDTA and preincubated at the same temperature for 5 min. Reoxidation was initiated by the addition of 0.025 volume of 20.5 mM GSSG and proceeded at the same temperature. The reoxidation was monitored by polyacrylamide gel electrophoresis after a two-step alkaline treatment procedure as described previously (19). In brief, reoxidized proteins were alkylated with 50 mM iodoacetic acid in the first step, precipitated in a cold acetone/HCl solution, dissolved in a urea solution, alkylated with 50 mM iodoacetamide in the second step, and then electrophoresed on an acid/urea gel.

For examining the iron-binding capacity of reoxidized half-molecule of ovotransferrin, we reoxidized the reduced protein for 22 h at 0 or 6 °C and trapped free sulfhydryls with 15 mM iodoacetamide. The sample was dialyzed and concentrated as described (17). In duplicate experiments, overall protein recovery was 40-55% at 0 °C (referred to the column of renaturation and 60-66% at 6 °C of renaturation. The reoxidized proteins were analyzed for the iron-binding capacity by urea-polyacrylamide gel electrophoresis as described (17). Alternatively, the iron-binding capacity was analyzed by a newly developed HPLC technique using an ion-exchange column (Mono-Q HR-5/5, Pharmacia LKB Biotechnology Inc.) jointed to a HPLC apparatus (Shimadzu, LC-4A). The native and reoxidized proteins were incubated at 0.62 mg/ml in 0.12 mM iron-nitritotriacetate complex at 0 °C for 10 min in 50 mM Tris-HCl buffer, pH 8.0, containing 4 mM sodium oxalate in a total volume of 0.08 ml, then mixed with 0.006 ml of 10 M urea. After incubation for 10 min, the sample was applied to the column equilibrated with 50 mM Tris-HCl, pH 8.0, containing 4.5 M urea. Protein was eluted at 35 °C with a linear gradient of 50-300 mM of the buffer and detected by absorbance at 280 nm (protein) and at 465 nm (iron-protein complex).

The abbreviations used are: GdnHCl, guanidine hydrochloride; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol (the reduced form); HPLC, high performance liquid chromatography.

RESULTS

Effects of Temperature on CD Spectrum of the Reduced Half-molecule—Disulfide-reduced proteins usually tend to aggregate. Although this is also true with the reduced ovotransferrin, our previous study has shown that it can be solubilized at low temperatures and at low protein concentrations (17). We have also shown that the CD spectrum of the fully reduced form of whole ovotransferrin, which is different from those of native and denatured forms, is variable depending on temperature conditions (17). We examined by using the isolated N-terminal and C-terminal half-molecules at 0.06 mg/ml, which lobe, the N-terminal or C-terminal lobe, is responsible for the variable CD spectrum. As shown in Fig. 1, the CD spectrum of the reduced N-terminal half-molecule was quite variable depending on temperature conditions, while the temperature-dependent variability was not evident with the C-terminal half-molecule. The variable CD spectrum observed with whole ovotransferrin, therefore, can be attributed to the N-terminal lobe of ovotransferrin.

Another important observation with the reduced N-terminal half-molecule was that the variable CD spectra give a clear isosbestic point at 215 nm. The variable CD spectra having an isosbestic point at 215 nm was also observed at twice the concentration (0.12 mg/ml) and half the concentration (0.03 mg/ml) of the reduced protein (data not shown). We, therefore, concluded that the variable CD spectrum is accounted for by a conformational transition but not by nonspecific aggregation.

We have examined whether the conformational transition can be explained by Scheme a, in which the native-like N$\text{S}_{\text{SH}}$ and fully denatured D$\text{S}_{\text{SH}}$ are included as the extreme states. As shown in Fig. 1, the CD spectrum of native, disulfide-bonded N-terminal half-molecule was quite different from those of the reduced form and did not intersect the isosbestic point. In the presence of 4.5 M urea, the CD spectrum of the reduced half-molecule, which shows a typical profile of fully denatured proteins, did not intersect the isosbestic point either (Fig. 1). These data were consistent with another transition scheme, in which the conformational transition of the reduced half-molecule occurs between some unique states.

![Fig. 1. Far ultraviolet CD spectra of the reduced N-terminal and C-terminal half-molecules. The reduced, denatured N-terminal half-molecule (left panel) and C-terminal half-molecule (right panel) were diluted at 6 (solid line, 1), 4 (solid line, 2) 2 (solid line, 3), or 0 °C (solid line, 4) with buffer B containing 0.5 mM DTT in a final protein concentration of 0.06 mg/ml. After 5 min of preincubation, the CD spectra were determined at the same temperature. The CD spectra of the native half-molecules in buffer B (dash-dotted line) and the reduced proteins in buffer B containing 0.5 mM DTT and 4.5 M urea (dotted line) were determined at 6 °C.](image-url)
that can be distinguished from $N_{\text{SH}}$ and $D_{\text{SH}}$.

$$
D_{\text{SH}} = \begin{bmatrix}
I_{\text{USH}} \\
I_{\text{FSH}}
\end{bmatrix}
$$

where $I_{\text{PSH}}$ is an intermediary state that is someway folded but differentiated from the native state, and $I_{\text{SH}}$ is a less folded intermediate that is different from $D_{\text{SH}}$. In this scheme, $D_{\text{SH}}$, induced in the presence of a denaturant can be converted by dilution into different ratios of $I_{\text{SH}}$ and $I_{\text{USH}}$ depending on temperature conditions.

To examine the temperature effects on the occurrence of $I_{\text{SH}}$ and $I_{\text{USH}}$, we determined protein ellipticity at 222 nm at different temperatures. As shown in Fig. 2, the ellipticity of the reduced form in the absence of urea was variable depending on temperature, while those of the reduced form in the presence of urea and of the native disulfide-bonded form were invariant under the tested temperature conditions. The absolute value of ellipticity was almost constant at a temperature range from 6 to 10 °C; but at temperatures lower than 4 °C it continued to decrease with lowering temperature. Although the ellipticity could not be determined at a wider temperature range because of the freezing problem at subzero temperatures and of the aggregation problem at temperatures higher than 10 °C, the data in Fig. 2 are consistent with the idea that the reduced half-molecule takes $I_{\text{FSH}}$ state at a temperature as high as 6 °C.

Reversibility of the Variable Conformation—It was examined whether or not the conversion between $I_{\text{FSH}}$ and $I_{\text{USH}}$ is reversible with each other. The preceding data show that the CD spectrum of the reduced protein corresponds to $I_{\text{FSH}}$ at 6 °C and probably to $I_{\text{SH}}$ at 0 °C. We examined the effects of temperature shifts from 0 to 6 °C or from 6 to 0 °C on the CD spectrum. As shown in Fig. 3, when the reduced form preincubated at 0 °C for 5 min was then incubated at a higher temperature of 6 °C for 1 h, the protein showed a CD spectrum very similar to the profile at 6 °C without the preincubation. Thus, the conversion from $I_{\text{USH}}$ into $I_{\text{FSH}}$ may be completed within 1 h of the incubation at 6 °C. In contrast, when the reduced half-molecule preincubated at 6 °C for 5 min was incubated at 0 °C for 5 h, the protein still retained CD spectrum very similar to the profile at 6 °C. It is not clear why the conversion from $I_{\text{USH}}$ to $I_{\text{FSH}}$ was not practically observed after a 5 h-incubation. A possible explanation is that the rate for the conversion form $I_{\text{USH}}$ to $I_{\text{USH}}$ is very slow at 0 °C.

Conformational Stability of the Reduced Half-molecule—The preceding data suggest that the intermediary CD spectra of the fully reduced N-terminal half-molecule can be accounted for by some partially folded conformation ($I_{\text{PSH}}$) rather than by the mixture of $N_{\text{SH}}$ and $D_{\text{SH}}$. To confirm this, we examined the stability of the folded conformation. No stable intermediate species is included in Scheme a; in Scheme b there is a possibility that $I_{\text{PSH}}$ can be experimentally detected as a stable state. The reduced protein was preincubated at 0 or 6 °C in non-denaturing buffer and then incubated at the same temperature in the presence of varying concentrations of a denaturant, before the ellipticity at 222 nm was determined. The denaturation profile was compared with the native, disulfide-bonded protein. As a denaturant, we employed GdnHCl, since upon the denaturation of very unstable mutant proteins, plots of $\Delta G$ versus denaturant concentrations have been shown to be linear with GdnHCl but not with other denaturants, including urea (20).

As shown in Fig. 4, the native, disulfide-bonded protein denatured with high cooperativity both at 0 and 6 °C; around 1.5 M GdnHCl sharp transition was observed. In contrast, the denaturation profiles of the reduced protein showed less cooperativity and at a much lower GdnHCl concentration transition occurred. When the unfolding of the reduced protein was compared with the two temperature conditions, the reduced protein appeared more stable at 6 °C.

To determine thermodynamic constants for denaturation, we used a two-state transition approximation and calculated the free energy change, $\Delta G_D$ for the reaction $F(\text{folded}) \rightleftharpoons U(\text{unfolded})$ at a given GdnHCl concentration using the equation

$$
\Delta G_D = -RT \ln \frac{\theta_f - \theta}{\theta - \theta_i}
$$

where $\theta$ is the observed ellipticity at 222 nm, and $\theta_f$ and $\theta_i$ are the ellipticities of the folded and unfolded states, respec-
Partly Folded State of Disulfide-reduced Ovotransferrin

Fig. 4. Effects of GdnHCl concentrations on the ellipticity of the N-terminal half-molecule. The reduced, denatured form (●) and the native form (□) of the N-terminal half-molecule were diluted at 6 °C (panel A) or 0 °C (panel B), preincubated at the same temperature for 5 min, then mixed with different concentrations of GdnHCl. After 7 min of incubation, the ellipticity at 222 nm was determined. In the insets, free energy for denaturation ΔG₀ was calculated as described in the text, and plotted as a function of GdnHCl concentration.

Fig. 5. Analyses of reoxidation of the reduced N-terminal half-molecule and of the iron-binding capacity of the reoxidized protein by PAGE. In panel A, the reduced, denatured N-terminal half-molecule was diluted at 6 °C, preincubated for 5 min, and then reoxidized at the same temperature for different times: lane 2, 0 min; lane 3, 2.5 min; lane 4, 5 min; lane 5, 10 min; lane 6, 20 min; lane 7, 40 min; lane 8, 1 h; lane 9, 5 h; lane 10, 5 h; lane 11, 22 h. The protein was alkylated by the two-step procedure and electrophoresed on an acrylamide gel. In lanes 1 and 12, the standard protein with different numbers of cleaved disulfide bonds was electrophoresed. The numbers to the right of lane 12 represent the numbers of disulfide bonds/protein molecule. In panel B, the native protein (lanes 1 and 2) and the protein reoxidized for 22 h at 0 °C (lanes 3 and 4) or at 6 °C (lanes 5 and 6) were analyzed for their iron-binding capacities by PAGE in the presence of 4.5 mM urea. Prior to electrophoresis, the proteins were incubated at 0 °C for 5 min in the presence (lanes 2, 4, and 6) or absence (lanes 1, 3, and 5) of 0.5 mM of FeCl₃-nitrotriacetate complex.

TABLE I

<table>
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<th>Protein form</th>
<th>Temperature (°C)</th>
<th>Linear method* ( \Delta G^0 ) (kcal/mol)</th>
<th>Binding method* ( \Delta G^0 ) (kcal/mol)</th>
<th>Molar activity (( k_a ))</th>
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</thead>
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<td>5.1</td>
<td>17.9</td>
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<tr>
<td></td>
<td>6</td>
<td>5.3</td>
<td>4.0</td>
<td>18.4</td>
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<tr>
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<td>1.0</td>
<td>2.2</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
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<td>2.2</td>
<td>2.6</td>
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* Calculated from the data in Fig. 4 based on Equation 2 (linear method) and Equation 3 (binding method) in the text.
6 °C, protein species with three disulfide bonds were predominant at 40 min of the reoxidation. At 22-h incubation, however, most, if not all, of the half-molecules of ovotransferrin finished for their disulfide bond formation. Essentially the same result was obtained when the reduced form was preincubated and reoxidized at 0 °C, except that protein species with three disulfide bonds was predominant at 1-h incubation (data not shown).

The fidelity of reoxidation was evaluated by a protein function, the iron-binding capacity. The iron-binding capacity was analyzed by PAGE in the presence of urea. Fig. 5 shows that both the holo-form and the apo-form of the reoxidized proteins have the same mobilities as the native half-molecule. These data qualitatively indicated that the reoxidized form is correctly renatured both at 0 and 6 °C in terms of iron-binding capacity. The band intensity of the holo-form, however, appeared significantly lower with the protein reoxidized at 0 °C than the native protein.

To determine the holo-form more quantitatively, we analyzed the native and reoxidized proteins by a HPLC technique. As shown in Fig. 6, native apotransferrin eluted at a retention time of 14.6 min, while the holo-form of the native protein eluted at a retention time of 25.7 min. When the partially iron-saturated native protein was analyzed, both the peaks by absorbance at 280 nm but only the peak of the holo-form by absorbance at 465 nm was detected (data not shown). These data demonstrated that the apo-form and the holo-form can be clearly separated on the HPLC and thus that the holo-form can be quantitatively determined from the peak area. No separation of the two forms was achieved when 4.5 M urea was excluded from the sample and elution buffers (data not shown). Thus, the separation of the two forms may be accounted for by that 4.5 M urea induces denaturation in the apoform but not in the holo-form.

The same amounts of the native and reoxidized proteins were analyzed by the HPLC technique. The proteins reoxidized at 0 and 6 °C were both eluted at the retention time of the holo-form as detected by absorbance at 280 and 465 nm. The peak area of the holo-form, as determined by absorbance at 465 nm, was 88 ± 2% (n = 2) with the protein reoxidized at 6 °C and 64 ± 2% (n = 2) with the one reoxidized at 0 °C, compared with the holo-form of the native protein. We, therefore, concluded that more efficient renaturation can be achieved at 6 rather than at 0 °C.

**DISCUSSION**

The experiments described here demonstrate that the renaturation pathway of the reduced, denatured N-terminal half-molecule of ovotransferrin can be explained by an extended form of Scheme b:

\[
\text{D}_{\text{SH}} \rightarrow \text{I} \rightarrow \text{N}_{\text{SH}} \rightarrow \text{F}_{\text{SH}}
\]

where \(N_{\text{SH}}\) represents the native, disulfide bonded protein. The reduced, denatured protein, \(D_{\text{SH}}\), can partially fold into \(I_{\text{SH}}\) (6 °C) or into a mixture consisting of \(I_{\text{SH}}\) and \(I_{\text{SH}}\) (0 °C). Partially folded \(I_{\text{SH}}\) that is a metastable state with \(\Delta G_0\) value of 2.2–2.8 kcal/mol again denatures upon the addition of a denaturant. The conversion from \(I_{\text{SH}}\) to \(I_{\text{SH}}\) is completed at 6 °C in 1 h, but the reverse might be so slow at 0 °C that little conversion can be detected in 5 h of incubation. The state \(I_{\text{SH}}\) that is distinguished from \(D_{\text{SH}}\) is assumed depending on the observation that the CD spectrum of \(D_{\text{SH}}\) does not intersect the isosbestic point (Fig. 1). The assumption may be reinforced by the following data. We have observed that the rate of \(D_{\text{SH}}\) to \(I_{\text{SH}}\) is very rapid and was completed within the dead time of sample mixing and the response time of a CD apparatus (about 15 s), but that at 30 min of incubation after a temperature shift from 0 to 6 °C, the conversion from \(I_{\text{SH}}\) to \(I_{\text{SH}}\) is still not completed. If \(I_{\text{SH}}\) were the same state as \(D_{\text{SH}}\), the rates for the conversions observed with the two different ways should have been identical. The high recovery of iron-bound form (86%) by the renaturation at 6 °C is indicative that \(I_{\text{SH}}\) is the immediate precursor to subsequent oxidative refolding processes. It is not clear whether at 0 °C of the renaturation, \(I_{\text{SH}}\) undergoes directly or via \(I_{\text{SH}}\) to oxidative refolding processes. By either possibility, our previous renaturation procedure that includes a temperature shift from 0 to 22 °C (17) appears reasonable in the light of Scheme c, since \(D_{\text{SH}}\) can be converted to \(I_{\text{SH}}\) + \(I_{\text{SH}}\) at 0 °C preincubation, and then during 22 °C reoxidation \(I_{\text{SH}}\) may be converted to \(I_{\text{SH}}\). The reoxidation at a high temperature of 22 °C should be efficient conditions for the covalent process of disulfide formation.

The renaturation pathway of ovotransferrin that includes a partially folded state of the disulfide-reduced form appears to be a unique model system for the investigation of protein folding. Most of small disulfide proteins that have been extensively investigated for oxidative refolding mechanisms take the denatured or native-like conformation in their disul-
fide-reduced states. No conformational alteration is required during the disulfide formation in the constant fragment of IgG light chain that has, in its reduced state, a conformation indistinguishable from the native form (6, 22). In contrast, bovine pancreatic trypsin inhibitor and RNase A take the denatured, random conformation in their fully reduced state (8, 9); folded intermediates can be recognized during reoxidation processes (3–5, 23). Other small disulfide proteins including RNase T1 (24), lysozyme (25), and trypsinogen (26) renature in a way similar to the latter case. Although the reduced RNase T1 takes native-like conformation in the presence of a high concentration of salt or at low temperatures (10–12), the renaturation pathway has not been elucidated under these conditions.

As a partially unfolded state, a “molten globule state” has been demonstrated with several protein examples (27–30). The molten globule is a state that takes some compact and native-like secondary structure and that is differentiated from the native state by the absence of close packing throughout the molecule and by a substantial increase in side chain’s fluctuations (27–30). The state can be induced under mild denaturing conditions such as acid, and its stability markedly depends on salt concentration (28–31). The molten globule state is also formed during refolding of denatured state, D, to native form, N, as an intermediate, A (30, 32, 33).

\[ D \rightleftharpoons A \rightleftharpoons N \]

The first step is completed within a ms time scale for α-lactalbumin, while the second stage involves a much slower rate-limiting process (30, 32). The renaturation process of the reduced N-terminal half-molecule of ovotransferrin is similar to the refolding scheme of α-lactalbumin. The state IF(SH) takes a metastable conformation that is different from the native or denatured state. The rate for the formation of IF(SH) from D(ISH) is very rapid and complete within 15 s of dead time for CD measurement; the rate for the IF(SH) to N(IS,SH) process is quite slow, so that incubation time as long as 22 h is required at 6°C. The major difference is that our renaturation system includes the covalent process of the disulfide formation. This allows us, by setting redox conditions, to separate the two sequential renaturation stages distinctly and also to analyze the intermediate state as a metastable conformation without using extreme conditions such as acid.

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