Renaturation of Ovotransferrin Under Two-step Conditions Allowing Primary Folding of the Fully Reduced Form and the Subsequent Regeneration of the Intramolecular Disulfides*

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A two-step procedure was found to be useful for the efficient refolding of a complex protein, ovotransferrin. In the first step, the reduced and denatured form of the protein was incubated at a low temperature in a non-denaturing buffer containing reduced glutathione; in the second step, the reduced form was reoxidized at a higher temperature in the presence of oxidized glutathione. Under these conditions, the fully reduced forms of ovotransferrin and its half-molecules were almost quantitatively reoxidized to regain iron-binding abilities and conformations, very similar to the native form. The circular dichroism spectra revealed that at low temperatures the fully reduced forms have partially folded conformations, which are fluctuating like “molten globule” states. The reoxidization kinetics compared between whole ovotransferrin and the two half-molecules supported independent refolding of the N- and C-terminal domains.

Studies of the mechanism for disulfide bond formation during protein folding are important not only theoretically, but also practically, because in many instances the regeneration of correct disulfide bonds is required for the recovery of the functional forms of many useful recombinant proteins produced in bacterial cells (Marston, 1986). The mechanism has been investigated in detail with relatively small proteins, such as bovine pancreatic trypsin inhibitor (Creighton, 1977; Creighton and Goldenberg, 1984), lysozyme (Acharya and Taniguchi, 1982), ribonuclease (Pace and Creighton, 1986; Scheraga et al., 1987), and the constant fragment of immunoglobulin light chain (Goto and Hamaguchi, 1981). Except for the renaturation studies of the individual domains of bovine serum albumin (Teale and Benjamin, 1977; Johanson et al., 1981), little is known about the refolding mechanism of a large protein that consists of a complex multidomain structure and many disulfide bonds.

Ovotransferrin consists of a single polypeptide chain with a molecular mass of about 78,000 Da, and contains 15 intramolecular disulfide bonds (Williams et al., 1982). The N-terminal and C-terminal halves of the chain form independent compact domains, each containing one specific iron-binding site (Williams et al., 1982). The iron-binding ability of each domain can be distinctly analyzed by a conventional PAGE, using whole ovotransferrin or the isolated half-molecules that correspond to the individual domains (Williams et al., 1978; Oe et al., 1988). Ovotransferrin appears therefore to be a useful model for the investigation of the refolding mechanism of a complex protein. Possible experimental difficulties are that the greater possibility for incorrectly paired disulfides could result in the formation of scrambled intermediates during the refolding process. In addition, upon disulfide re-duction ovotransferrin makes aggregates through intermolecular hydrophobic interactions, thereby forming a hard gel (Hirose et al., 1986).

In the present study, we have searched for refolding conditions in which the aggregation of the reduced form can be avoided and the formation of scrambled intermediates can be skipped. Our idealized refolding scheme has been that the reduced form is folded into a native-like conformation, before the conformation is fixed or improved by the regeneration of the intramolecular disulfide bonds. The loss of conformational entropy upon folding of a reduced form should be decreased at a lower temperature; in contrast, the covalent process of disulfide formation should proceed more rapidly at a higher temperature. To conform the different requisites, we have separated the refolding process into two steps: the first step has included reduced and low temperature conditions, and the second step, oxidized and higher temperature conditions. We show that under the two-step conditions, the fully reduced forms of ovotransferrin and its half-molecules can be quantitatively reoxidized to regain the iron-binding ability and native-like conformation without making a substantial aggregate. Reoxidization kinetics compared between whole ovotransferrin and the two half-molecules supports independent folding of the N-terminal and the C-terminal domain.

EXPERIMENTAL PROCEDURES

Materials—Ovotransferrin and its half-molecules (N-terminal and C-terminal half-molecules) were prepared as described (Oe et al., 1988). Reduced and oxidized glutathione were purchased from Dojin. Other chemicals were from Nacalai Tesque.

Alkylated trypsin inhibitor was used as a carrier for protein precipitation by acetone. Soybean trypsin inhibitor (Sigma, Type I-S) was fully reduced by the incubation with 10 mM DTT in Buffer A (8 M urea, 50 mM Tris-HCl, pH 8.2, 1.0 mM Na-EDTA) at 37 °C for 30 min, and then alkylated with 50 mM iodoacetic acid at 37 °C for 15 min in Buffer A. The sample was passed through a Sephadex G-25 column (NAP-25, Pharmacia LKB Biotechnology Inc.) equilibrated with 25 mM sodium phosphate buffer, pH 7.0.

Denaturation and Reduction of Ovotransferrin—Reduced, denatured proteins were prepared by the incubation of whole ovotransferrin and its half-molecules with 5.0 mM DTT at 37 °C for 30 min in

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‡ This abbreviation was used: PAGE, polyacrylamide gel electrophoresis; GSH, the reduced form of glutathione; GSGG, the oxidized form of glutathione; DTT, diithiothreitol (the reduced form).
Diluted 100-fold at 0 °C with Buffer B containing 1.0 mM GSH, and being washed in the same way, the proteins were dissolved in 10 mM trophotometric method using 4,4'-dithiopyridine (Grassetti and Mur-  estimated from the amounts of free sulfhydryls measured by a spec-

Progressed at 22 °C with 0.25 mM 4,4'-dithiopyridine in the same buffer containing 7.5 M acid/urea/PAGE and the preparation of standard proteins for 6 min. Acid/urea/PAGE and the preparation of standard proteins can be distinctly detected by this technique. Briefly, the reoxidization was terminated by trapping free sulfhydryls with an alkylation re-

Three-step alkylation and PAGE (Hirose et al., 1988), since protein species with different numbers of disulfide bonds can be distinctly detected by this technique. Briefly, the reoxidization was terminated by trapping free sulfhydryls with an alkylation re-

Measurement of CD Spectra—Ovotransferrin and its half-molecu- cules in their native, reoxidized, or fully reduced forms were analyzed for CD spectra. The reoxidized form of whole ovotransferrin was prepared in the same way as described for iron-binding analyses. The reduced, denatured forms (15 mg/ml) of whole ovotransferrin and its half-molecules were diluted 100-fold at the indicated temperatures with 25 mM Tris-HCl, pH 8.2, containing 0.25 mM Na-EDTA and 0.5 mM DTT. CD spectra were measured using a cuvette of 0.1-cm path length with a Jasco J-50IC spectropolarimeter attached with a data processor (Jasco DP-501). The temperature of the cuvette was maintained with a circulating water bath. Analyses of CD spectra for the native and reoxidized forms were performed at a protein concentration of 0.15 mg/ml at room temperature in 25 mM Tris-HCl, pH 8.2, containing 0.25 mM Na-EDTA, 0.5 mM DTT, and 8 M urea. The CD spectra were measured at least three times on different days and the results were averaged. The data were expressed in mean residue ellipticity (degree cm⁻²/dmol), using the mean residue weights of 110 for whole ovotransferrin and the N-terminal half-molecule, and 111 for the C-terminal half-molecule.

RESULTS

Solubility of the Reduced Form of Whole Ovotransferrin—We examined protein solubility during refolding under different temperature and redox conditions by monitoring turbidity of a mixture containing reduced whole ovotransferrin. When reduced, denatured ovotransferrin was diluted 100-fold at 30 °C with Buffer B containing 1.0 mM GSH and incubated under the same buffer and temperature conditions, turbidity appeared in a short incubation time (Fig. 1A). The same was also true when the buffer contained 1.0 mM GSH and 0.5 mM GSSG. However, when the reduced, denatured protein was diluted at 0 °C with Buffer B containing 1.0 mM GSH and preincubated at the same temperature for 5 min, the turbidity appearance during subsequent incubation at 30 °C was significantly decreased both in the presence and absence of GSSG (Fig. 1A). During the incubation at a lower temperature, 15 °C, the reduced protein preincubated at 0 °C showed no turbidity in the presence and absence of GSSG, while turbid-

FIG. 1. Aggregation of the reduced form of whole ovotransferrin. In A-C, the reduced, denatured whole ovotransferrin was diluted 100-fold at 30 (A), 22 (B), or 15 °C (C) with Buffer B containing 1.0 mM GSH (C) or 1.0 mM GSH and 0.5 mM GSSG (B). Alternatively, the reduced, denatured protein was diluted 100-fold at 22 °C with Buffer B containing 1.0 mM GSH, preincubated at 0 °C for 5 min in the same buffer, then incubated at 30 (A), 22 (B), or 15 °C (C) in Buffer B containing 1.0 mM GSH (C), or 1.0 mM GSH and 0.5 mM GSSG (B). Aggregation was monitored by recording the absorbance at 490 nm. In D, the diluted protein was preincubated at 0 °C for 15 s, 1 min, or 15 (min) in Buffer B containing 1.0 mM GSH and then incubated at 22 °C in Buffer B containing 1.0 mM GSH and 0.5 mM GSSG.

The formation of disulfide bonds in some protein samples was also estimated from the amounts of free sulfhydryls measured by a spectrophotometric method using 4,4'-dihydroxyphenylalanine (Reddy and Mur-
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The nature of reduced ovotransferrin is modulated in such a way that intermolecular aggregation during the subsequent incubation at higher temperatures is markedly decreased. An intermediate temperature, 22 °C, the preincubated protein showed no turbidity in the presence of GSSG, but in the absence of GSSG it showed significant turbidity after 5 min of incubation (Fig. 1B). Therefore, the soluble property of reduced ovotransferrin gained by preincubation at 0 °C may be stabilized at 22 °C in the presence of GSSG.

To examine the effects of preincubation time, we preincubated the reduced ovotransferrin at 0 °C for different times in Buffer B containing 1.0 mM GSH and recorded the turbidity appearance at 22 °C in Buffer B containing 1.0 mM GSH and 0.5 mM GSSG. As shown in Fig. 1D, the preincubation of at least 1 min was required for preventing turbidity appearance at 22 °C.

The Effects of Redox Conditions on Disulfide Bond Formation—Protein aggregation must be avoided in refolding studies; but higher temperature conditions are desirable for a covalent process including disulfide bond formation. Thus, the reduced proteins were reoxidized at 22 °C in the presence of GSH and GSSG, after the preincubation at 0 °C in the presence of GSH only.

The extent of protein disulfide formation at an equilibrium should vary not only with the GSH to GSSG ratio but also with the absolute concentration of GSH: it should increase as the absolute concentration of GSH decreases (Walters and Gilbert, 1986). The formation of disulfide bonds in whole ovotransferrin and its half-molecules was analyzed after a long reoxidation time in the presence of different absolute concentrations of GSSG, as well as in the presence of different molar ratios of GSSG to GSH.

When the refolding buffer contained 1.0 mM GSH and 0.3–0.5 mM GSSG, almost all of the N- and C-terminal half-molecules were completed for their disulfide bond formation during 16 h of the reoxidization (Fig. 2). But in the presence of 10 mM GSH, significant amounts of the proteins with free sulfhydryls still remained at the same GSSG to GSH ratios.

Disulfide bond formation in whole ovotransferrin was examined in the presence of 1.0 mM GSH and different concentrations of GSSG. In the presence of 1.0 mM GSH and 0.5 mM GSSG, disulfide bonds appeared to be almost completely formed in whole ovotransferrin (Fig. 2). With decreasing the concentration of GSSG, the extent of disulfide bond formation appeared to be decreased (Fig. 2). The results by PAGE were confirmed by the spectrophotometric method using 4,4'-ditiopyridine, which can determine free protein sulfhydryls. When the reduced protein was reoxidized in the presence of 1.0 mM GSH and the same range of concentrations of GSSG, the averaged numbers of free sulfhydryls per mol of the protein, determined in four to eight experiments, were 5.7 ± 0.5 (SE) in 0.05 mM GSSG, 1.8 ± 0.3 in 0.1 mM GSSG, 1.2 ± 0.2 in 0.2 mM GSSG, 0.3 ± 0.2 in 0.3 mM GSSG, and 0.2 ± 0.2 in 0.5 mM GSSG.

Iron-binding Capacity of Reoxidized Ovotransferrin—We evaluated the functional property of the reoxidized proteins by their iron-binding abilities. The iron-binding ability of ovotransferrin was analyzed by PAGE in the presence of urea, which induces denaturation in the ligand-free form but not in the iron-bound form. The major advantage of this technique is that the iron-binding abilities of the two domains in whole ovotransferrin can be distinctly analyzed (Evans and Williams, 1980). As shown in Fig. 3, the mobility of native ovotransferrin was increased with increasing the molar ratio of iron to protein. In the presence of an excess concentration of iron, the protein took the d Ferric form only. At partial saturation levels with iron, the two monoferric forms (i.e., FeOVT, the monoferric form with iron in the N-terminal domain site; OVTFe, the monoferric form with iron in the C-terminal domain site) were detected. With regard to the reoxidized ovotransferrin, some protein species, probably a polymerized form, remained on the top of the gel. However, almost all the protein species that entered the gel showed the same mobilities as native ovotransferrin at all the saturation levels with iron.

Similar analyses were carried out with the reoxidized half-molecules. Little protein remained on the top of the gel; almost all of the reoxidized species showed the same mobility as the native half-molecules both in the presence and absence of a saturated level of iron.

These data indicated that the reoxidized forms of whole ovotransferrin and its half-molecules were correctly refolded in terms of iron-binding capacity.

Kinetics of the Formation of Disulfide Bonds—The kinetic behavior of the disulfide bond formation was compared between whole ovotransferrin and the half-molecules. After the preincubation at 0 °C, the proteins were reoxidized for different times and analyzed for their disulfide bond formation by
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Fig. 3. Iron-binding abilities of the reoxidized forms of whole ovotransferrin and its two half-molecules. In A, the native (lanes 1–6) and the reoxidized form (lanes 7–12) of whole ovotransferrin were analyzed for their iron-binding capacities by PAGE in the presence of 6 M urea. Prior to electrophoresis, the proteins were incubated at 0 °C for 5 min at different molar ratios of iron (FeCl₃/nitrilotriacetate) to protein: lanes 1 and 7, 0; lanes 2 and 8, 0.5; lanes 3 and 9, 0.75; lanes 4 and 10, 1.0; lanes 5 and 11, 1.5; lanes 6 and 12, 10. OT, OTFe, FeOT, and FeOT represent the iron-free form, the monoferric form with iron in the C-terminal domain, the monoferric form with iron in the N-terminal domain, and the diferric form of ovotransferrin, respectively. In B, the native forms of the N-terminal half-molecule (lanes 1 and 2) and the C-terminal half-molecule (lanes 3 and 4) and the reoxidized forms of the N-terminal half (lanes 5 and 6) and the C-terminal half (lanes 7 and 8) were incubated at 0 °C for 5 min in the presence (lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of 0.5 mM of FeCl₃-nitrilotriacetate complex and then electrophoresed in the presence of 4.5 M urea.

PAGE. As shown in Fig. 4A, the protein species with greater numbers of disulfide bonds increased with the incubation time. The electrophoresis patterns appeared to exclude such an extreme reoxidation pathway in which there is a strong rate-limiting step, leading to the accumulation of an intermediate with a specified number of disulfide bonds.

We analyzed the reoxidation rates quantitatively by determining the amounts of free sulfhydryls by the spectrophotometric method. Semilogarithm plots of the time courses revealed that the disulfide formation in all the ovotransferrin forms proceeded in first order until 13 min incubation time (Fig. 4B). Apparent first order rate constants determined from the slopes were 0.14 min⁻¹, 0.077 min⁻¹, and 0.058 min⁻¹ for the N-terminal half-molecule, whole ovotransferrin, and the C-terminal half-molecule, respectively. The sum of sulfhydryls in the two half-molecules at each reoxidation time was almost exactly the same as the value of whole ovotransferrin. There is no disulfide bridge between the N- and C-terminal domain in native ovotransferrin (Williams et al., 1982). Therefore, it is unlikely that the formation of interdomain disulfides, which would delay overall disulfide formation, is involved in the reoxidation pathway of whole ovotransferrin.

CD Spectrum—The preceding data indicate that preincubation at 0 °C of the reduced forms is important for the renaturation of ovotransferrin and its half-molecules. We monitored folding of the reduced forms of whole ovotransferrin and its half-molecules by analyzing CD spectra at low temperatures. At 0 °C the spectrum of reduced whole ovotransferrin showed a sharp negative peak at 204 nm and a broad shoulder at around 220 nm (Fig. 5A). Similar profiles were observed with the reduced forms of the N- and C-terminal half-molecules (Fig. 5B). These profiles were not identical with those of the native forms, but they were quite different from the spectrum of the denatured form of whole ovotransferrin determined in the presence of 8 M urea. The CD spectrum of the reoxidized whole ovotransferrin showed a profile, very similar to the native form. These data were consistent with the idea that both the N- and C-terminal domains in whole ovotransferrin take partially folded conformations during the preincubation, and that the conformations can be improved by the reoxidization of sulfhydryls.

The reoxidization step included a temperature shift. The stability of the folded conformation was evaluated by the CD spectrum of reduced whole ovotransferrin at temperatures lower than 8 °C without preincubation at 0 °C, in which no aggregation was induced during measuring time of 10 min for CD spectrum. As shown in Fig. 5A, the CD spectrum varied significantly depending on temperature conditions. For example, the negative peak at 204 nm observed at 0 °C was less pronounced and shifted to 206 nm at 8 °C; the ellipticity at 220 nm was decreased with increasing temperatures. To examine whether the folded conformation at 0 °C is retained during the temperature shift, we diluted the reduced, denatured form of whole ovotransferrin at 0 °C with a nondenaturing buffer preincubated for 5 min at 0 °C, and incubated at an indicated temperature (0–22 °C) for 4 min before ellipiticity was measured at 205 and 220 nm. As shown in Fig. 5B, the ellipticity at 205 nm was greatly increased with increasing the temperature; in contrast, the value at 220 nm slightly decreased at higher temperatures. Therefore, after the temperature shift to 22 °C, the reduced form of whole ovotransferrin may take a folded conformation which is slightly different from the one formed during the preincubation at 0 °C.

DISCUSSION

That the amino acid sequence contains all the information required for the correct folding of a globular protein has been confirmed by in vitro renaturation studies of many proteins. However, other proteins, including proteins with multidomain structure and many disulfides, do not always fold efficiently into their original conformation in vitro. Purvis et al. (1987) have proposed an in vivo mechanism in which the efficiency of folding of these problematic proteins is increased by controlled rates of translation. An essential role of protein disulphide isomerase on the disulfide bond formation of secretory proteins in vivo has been demonstrated (Bulleid and Freedman, 1988). Ovotransferrin, a secretory protein synthesized in oviduct cells, is a complex multidomain protein containing...
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**Fig. 4.** Kinetics of the formation of disulfide bonds. In A, the reduced, denatured forms of the N-terminal half-molecule (N), the C-terminal half-molecule (C), and whole ovotransferrin (W) were diluted at 0 °C, preincubated for 5 min, and then reoxidized at 22 °C in Buffer B containing 1.0 mM GSH and 0.5 mM GSSG for different times (min): lane 2, 0; lane 3, 1; lane 4, 2; lane 5, 4; lane 6, 6; lane 7, 9; lane 8, 13; lane 9, 20; lane 10, 60; lane 11, 180. The numbers of disulfide bonds were analyzed by the two-step alkylation and PAGE techniques. The alkylation of the two half-molecules was performed with iodoacetamide in the first step and with iodoacetic acid in the second step. Whole ovotransferrin was alkylated with iodoacetic acid in the first step and with vinylpyridine in the second step. In B, the reduced forms of whole ovotransferrin (O), the N-terminal half-molecule (O), and the C-terminal half-molecule (C) were reoxidized in the same way. The numbers of free sulfhydryls per protein molecule were determined spectrophotometrically. x, represents the sums of the sulfhydryls of the two half-molecules.

15 intrachain disulfide bonds (Williams et al., 1982). Indeed, our early refolding study of ovotransferrin using a conventional one-step procedure was unsuccessful because of the insolubility of the reduced form. However, the present work demonstrates that after a brief preincubation at a low temperature, the reduced forms of whole ovotransferrin and its half-molecules can be reoxidized to regain the iron-binding ability without making substantial aggregates. Therefore, the in vitro mechanisms may not always be required for the correct refolding of a complex disulfide protein if an in vitro system involves some rationally devised conditions.

Ovotransferrin is a water-soluble protein in its native state; but in the fully reduced state, it makes aggregates under moderate temperature conditions (Hirose et al., 1986). The soluble property of reduced ovotransferrin at a low temperature can be explained by far-ultraviolet CD spectra which show that the reduced form takes a partially folded conformation at 0 °C: hydrophobic amino acid side chains, which would cause intermolecular interactions, could be buried into the interior of a folded conformation. Although we have not determined exactly the rate of the formation of a folded conformation, we have observed in a manual dilution experi-

**Fig. 5.** Far-ultraviolet CD spectra of different forms of ovotransferrin. A, the CD spectra of different forms of whole ovotransferrin (the native form, —; the reoxidized form, ——; the reduced form in the presence of 8 M urea, ——) were recorded at a room temperature. The reduced form of whole ovotransferrin under non-denaturing conditions (-----) was analyzed at 0 °C. B, the native forms of the N-terminal half-molecule (---) and the C-terminal half-molecule (-----) were analyzed at room temperature; the reduced forms of the N-terminal half (-----) and the C-terminal half (------) were analyzed at 0 °C under non-denaturing conditions.

**Fig. 6.** Effects of temperature on the CD spectra of the reduced form of whole ovotransferrin. In A, the reduced, denatured whole ovotransferrin was diluted at 2 °C, 6 °C (-----), or 8 °C (—--—) with Buffer B containing 0.5 mM dithiothreitol. The CD spectra were determined under the same buffer and temperature conditions. In B, the reduced, denatured whole ovotransferrin was diluted at 0 °C with the same buffer, and preincubated at the same temperature for 5 min. The mixtures were incubated at the indicated temperatures for 4 min, before mean residue ellipticity was determined at 205 (○) or 220 nm (○).
The sums of those in the two half-molecules (Fig. 1D) can therefore be related to the rate of partial folding of the reduced form.

The observation that the CD spectrum of the reduced form of ovotransferrin is variable depending on temperature conditions (Fig. 6) suggests the fluctuating nature of its folded conformation. A compact state with fluctuating tertiary structure, termed a "molten globule" state has been demonstrated with partly denatured proteins, including α-lactalbumin (Kuwajima et al., 1976; Dolgikh et al., 1981), cytochrome c (Ohgushi and Wada, 1983), the Fc fragment of immunoglobulin (Vonderviszt et al., 1987), and ovalbumin (Koseki et al., 1988). With respect to the fully reduced forms of disulfide proteins, bovine pancreatic trypsin inhibitor (Kosen et al., 1983) and RNase A (Galat et al., 1981) do not have such a compact conformation as evaluated by far-ultraviolet CD spectra. However, fully reduced RNase T₁ shows 53% of native activity at 5 °C (Pace et al., 1988); hen egg lysozyme has some secondary structure in its fully reduced form (White, 1982); the reduced form of the constant fragment of immunoglobulin light chain takes a less stable, but almost exactly the same, conformation as the native form (Goto and Hamaguchi, 1986). Furthermore, very recent studies using nonradiative excitation energy-transfer measurements have demonstrated that reduced bovine pancreatic trypsin inhibitor has a compact conformation (Amir and Haas, 1988). Therefore, molten globule-like conformations might be commonly related to the reduced forms of disulfide proteins.

The second step of the refolding involves a temperature shift and the incubation in the presence of GSSG. The CD spectrum of reduced ovotransferrin is variable at different temperatures, but the absolute value of the ellipticity at 220 nm that was observed at 0 °C is retained or even enhanced at 22 °C during at least 4 min incubation (Fig. 6). At 4 min incubation of the reoxidation, no fully reduced form of whole ovotransferrin remains (Fig. 4A, panel W, lane 5). Therefore, it is likely that a partially folded conformation formed at 0 °C is slightly modulated at 22 °C before the sequential formation of disulfide bonds stabilizes and improves the conformation.

The well characterized protein folding pathway determined with bovine pancreatic trypsin inhibitor includes the formation of incorrectly paired disulfide bonds as refolding intermediates and the subsequent reshuffles to reach the correct pairings (Creighton, 1977). At present, similar information is not available with ovotransferrin. However, the occurrence of an intermediate with an interdomain disulfide pairing, which would delay overall rates of disulfide formation, is unlikely, because during the reoxidization the amounts of free sulfhydryls in whole ovotransferrin are almost exactly the same as the sums of those in the two half-molecules (Fig. 4).

In conclusion, our two-step procedure may be useful in searching for suitable conditions for the correct refolding of natural and recombinant proteins. Poor recovery of functional forms often seen with proteins containing many disulfides could be attributed to the formation of scrambled forms during reoxidization. By the incubation under reduced conditions prior to reoxidization, incorrect pairing of sulfhydryls occurring at an early folding stage could be skipped. The two-step procedure may also be a useful experimental system for the study of the folding mechanism of a disulfide protein. Molten

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2 M. Hirose, T. Akuta, and N. Takahashi, unpublished data.