Oxidative Regeneration and Selective Reduction of Native Disulfide Bonds in the N-terminal Half-molecule of Ovotransferrin*

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The denatured, disulfide-reduced form of the N-terminal half-molecule of ovotransferrin was reoxidized with either oxidized diithiothreitol or GSSG and analyzed for the localization of disulfide bonds. Chemical analyses of the reoxidized proteins revealed that the disulfide peptides corresponding to the six native protein disulfides (SS-I, SS-II, SS-III, SS-IV, SS-V, and SS-VI) are all regained in the reoxidized protein. The peptide recoveries from the reoxidized proteins were, however, about half of those from the native protein with respect to the two inner disulfides (SS-IV and SS-V) in the kringle bridges, but all the disulfide peptides corresponding to the remaining disulfides (SS-I, SS-II, SS-III, and SS-VI) were recovered at almost equivalent yields in the native and reoxidized proteins. In addition, on searching for a nonnative disulfide peptide, the two disulfides, Cys177-Cys174 and Cys174-Cys182, which can be accounted for by mispaired bridges of sulphydrys in SS-IV and SS-V, were detected in the protein reoxidized with oxidized diithiothreitol. Upon disulfide reduction of the native protein with reduced diithiothreitol, both SS-IV and SS-V were selectively cleaved under the same buffer and temperature conditions as in the oxidative refolding. The lower stabilities of the two inner disulfide bonds in the kringle might be related to the lower recoveries of the disulfide peptides from SS-IV and SS-V and the generation of the nonnative disulfide bonds.

Oxidative refolding of a reduced disulfide protein is a useful model system for the investigation of protein folding mechanisms, because all disulfides present at the folding pathway can be trapped in stable forms (Creighton, 1986). In vitro, small single-domain proteins such as BPTI (Creighton, 1978; Weissman and Kim, 1991), lysozyme (Acharya and Taniuchi, 1982), ribonucleases (Pace and Creighton, 1986; Scheraga et al., 1987), and the constant fragment of immunoglobulin light chain (Goto and Hamaguchi, 1981) can be oxidatively refolded from their denatured, reduced states into the native, disulfide-bonded form. In BPTI, the well documented example, several different intermediate species can be trapped during oxidative refolding, and a folding pathway can be quantitatively elucidated (Creighton, 1978; Creighton and Goldenberg, 1984; Weissman and Kim, 1991, 1992). The folding mechanism is, however, poorly understood in more complex proteins in which the usefulness of an oxidative refolding system should be emphasized because of the limitation of a technical approach for conformational analysis.

Difficulty in the correct oxidative refolding in a complex protein is usually related to the low solubility of disulfide-reduced intermediates and/or to the formation of a scrambled form having incorrect disulfide bonds. The low solubility problem has been overcome by introducing glutathione-mixed disulfides into the free sulphydryls of denatured chymotrypsinogen (Light and Al-Obeidi, 1991) and trypsinogen (Higaki and Light, 1986) or by gradually decreasing a denaturant concentration during the renaturation of the gelatin-binding fragments of fibronectin (Ingham and Brew, 1992). The integrity of the reoxidized proteins, however, has been evaluated only functionally. The analysis for disulfide localization in the reoxidized proteins, which is a prerequisite for detailed study of folding pathways, has not been done.

As an alternative method, we have previously shown that the regaining of the iron binding capacities of ovotransferrin and of its N- and C-terminal half-molecules can be attained by a two-step procedure in which the denatured, reduced proteins, D(SH), are initially transformed into a partially folded state, I(SH), under reduced conditions and are then reoxidized with GSSG into the native, disulfide-bonded form, N(SS) (Hirose et al., 1989; Hirose and Yamashita, 1991).

\[ \text{D(SH)} \rightarrow \text{I(SH)} \rightarrow \text{N(SS)} \]

**SCHEME A**

Similar efficient oxidative refolding via the two-step procedure can also be observed with serum albumin and ovalbumin, which respectively assume a partially folded state and a native-like conformation in their disulfide-reduced forms (Lee and Hirose, 1992; Takahashi et al., 1991; Takahashi and Hirose, 1992), suggesting that such a two-step procedure generally works for efficient oxidative refolding of a disulfide protein that assumes a certain folded conformation under disulfide-reduced conditions.

In the present study, the denatured, reduced N-terminal half-molecule of ovotransferrin was reoxidized with DTTred as well as GSSG, and the localization of disulfide bonds in the reoxidized proteins was determined. The N-terminal half-molecule of ovotransferrin consists of two domains (Domains 1 and 2) as shown in Fig. 1. Domain 1 is formed by two nonconsecutive peptides, which contain two disulfide bonds (SS-I and SS-II); Domain 2 contains three disulfides consisting of kringle bridges (SS-III, SS-IV, and SS-V) and one additional disulfide (SS-VI) (Williams et al., 1982). Data from chemical analyses revealed that all the disulfide peptides corresponding to the six native disulfide bonds are regained in the reoxidized protein. The peptide recoveries from the
reoxidized proteins were, however, about half of those from the native protein with respect to the two inner disulfides (SS-IV and SS-V) in the kringle, but all the disulfide peptides corresponding to the remaining disulfides (SS-I, SS-II, SS-III, and SS-VI) were recovered in almost equivalent yields in the native and reoxidized proteins. In addition, nonnative disulfide peptides corresponding to mispaired disulfides derived from the two inner kringle disulfides were detected in the reoxidized protein. Upon disulfide reduction of the native protein with DTT, both the SS-IV and SS-V were selectively cleaved. The lower stabilities of the two inner disulfide bonds may be related to the lower recoveries of SS-IV and SS-V and also to the generation of nonnative disulfides.

**EXPERIMENTAL PROCEDURES**

**Materials**—The N-terminal half-molecule of ovotransferrin was prepared as described by Os et al. (1988). GSH and GSSG were purchased from Kohjin. Trypsin was purchased from Sigma (Type XI, treated with diphenylcarbamyl chloride). DTT was purchased from Calbiochem. SBD-F was obtained from Dojinco. Other chemicals were guaranteed grade from Nacalai Tesque.

**Reduction of Denatured, Reduced Protein**—The denatured, reduced N-terminal half-molecule of ovotransferrin was prepared by the incubation of the protein at 2.0 mg/ml with 5.0 mM DTT at 37 °C for 30 min in Buffer A (0.1 M Tris-HCl buffer, pH 8.2, 0.1 mM Na-EDTA) containing 8 M urea. The reoxidation with DTT was performed by a one-step procedure in which the denatured, reduced protein was diluted 100-fold at 6 °C with Buffer A containing various concentrations of DTT. After various time intervals at the same temperature, the protein was trapped with 50 mM iodoacetic acid, precipitated in cold acetone/HCl, dissolved in urea solution, fully reduced with DTT, alkylated with 50 mM iodoacetamide, and then electrophoresed on an acid-urea gel as described before (Hirose et al., 1988, 1989). In preliminary experiments on the oxidative refolding with DTT, we purified the commercially obtained DTT by the method described by Creighton (1984) and compared the purified and unpurified DTT preparations with respect to their ability to reoxidize the six disulfide bonds in the denatured, reduced half-molecule. The analysis by the PAGE technique revealed that the disulfide formation was almost completed within 22 h of reoxidation with the unpurified DTT but not with the purified material (five disulfide species as the major band). One may explain this by the presence of a less stable disulfide impurity in the unpurified DTT as pointed out by Creighton (1984). However, when the purified DTT was analyzed by HPLC using a Yvad reverse-phase C18 analytical column as described by Chau and Nelson (1991), the presence of several unidentified impurities that can be differentiated from either DTT, GSSG, or GSH was still detected in the purified DTT preparation. Thus, the cyclic disulfide purified by Creighton’s method was further purified by the same reverse-phase HPLC, except that the analytical column was replaced by a preparative column. In brief, 2 g of DTT was that had been purified as described by Creighton (1984) were dissolved in 85 ml of 0.1% trifluoroacetic acid, applied to a Cosmosil SC18-AR-300 column (20 × 250 mm), and eluted with 150 ml of 50% acetonitrile, 0.1% trifluoroacetic acid. The major DTT peak was collected and lyophilized. The denatured, reduced N-terminal half-molecule of ovotransferrin almost completely its disulfide bond formation in 22 h of reoxidation with 100 mM of the HPLC-purified DTT (see Fig. 2). Thus, the DTT preparation purified by the HPLC procedure was used for all experiments.

For the analysis of the localization of disulfide bonds, the denatured, reduced protein was reoxidized with 100 mM of the HPLC-purified DTT for 22 h under the same buffer and temperature conditions, trapped with iodoacetic acid, dialyzed, and concentrated in the same way as described before (Hirose et al., 1989). When GSSG was used as an oxidant, oxidative refolding was performed by a two-step procedure as described above (Hirose and Yamashita, 1991). In brief, the denatured, reduced protein was diluted 100-fold at 6 °C with Buffer A containing 1.0 mM GSH and preincubated for 5 min at the same temperature. Oxidation was initiated by the addition of 0.025 volume of 20.5 mM GSSG, giving a final concentration of 0.5 mM, and allowed to proceed at the same temperature for 22 h. The sample was trapped with iodoacetamide, dialyzed, and concentrated in the same way.

**Analysis of the Localization of Disulfide Bonds**—The reoxidized half-molecule or the native protein as a control was incubated at 0.54 mg/ml with trypsin in a protein to protease ratio of 40 (w/w) at 30 °C for 20 h in 50 mM Tris-HCl, pH 7.0, containing 5 mM CaCl2 and 2.0 mM urea in a total volume of 1.43 ml. Proteolysis was terminated by the addition of a 0.11 volume of 5% trifluoroacetic acid. The trypsin digest was fractionated by a reverse-phase HPLC column (YMC AP-302, ODS 46 × 150 mm, pore size, 30 nm) joined to a HPLC apparatus (Shimadzu, LC-5A). Peptides were eluted with an acetonitrile linear gradient (0-60%) in 0.1% trifluoroacetic acid. A part of each peak was assayed for disulfides by the method of Sueyoshi et al. (1985) using a fluorescent reagent, SBD-F. Fluorescence was measured with a Hitachi fluorescence spectrophotometer (model F-3000) with excitation at 385 nm and emission at 515 nm. Disulfide-positive peaks were further purified by rechromatography on the same column but with a different buffer system (0-60% acetonitrile gradient in 10 mM triethylamine/acetic acid buffer, pH 5.0). Purified disulfide peptides were analyzed for their amino acid compositions with an amino acid analyzer (Hitachi, model 835-30) and primary sequences with a gas-phase protein sequenator (Applied Biosystems, model 477A/120A). For the amino acid analysis, the purified peptides were hydrolyzed in the gas phase with 8 M hydrochloric acid containing 0.1% (v/v) phenol for 24 h at 110 °C under vacuum. Cysteine residues were determined as cystic acid after oxidation by performic acid as described by Moore (1963).

**Reduction of Native Disulfide Bonds under Nondenaturating Conditions**—The native half-molecule was incubated at 0.2 mg/ml in Buffer A with various concentrations of DTT at 6 °C for 30 min and alkylated with 50 mM iodoacetic acid at 37 °C for 5 min. The number of intrachain disulfide bonds was determined by the PAGE technique in the same way as described above. For the analysis of the localization of reduced disulfide bonds, the native protein was incubated for 30 min with 10 mM DTT under the same buffer and temperature conditions and alkylated with iodoacetic acid in the same way. The sample was concentrated using a concentrator (Amicon, Centricon-40, 0.5 ml, 30), passed through a Sephadex column (NAP-10, Pharmacia LKB Biotechnology Inc.), equilibrated with distilled water, applied to a Cosmosil SC18-AR-300 column (4.6 × 150 mm), and eluted by a linear 20-ml gradient of 30-80% acetonitrile in 0.1% trifluoroacetic acid. Proteins in two major peaks were further purified by rechromatography on the same column and buffer system and assayed for the number of disulfide bonds by the PAGE technique. The protein species having four disulfide bonds along with the native control were proteolyzed with trypsin, and peptide fragments were fractionated with reverse-phase HPLC, purified by rechromatography, and analyzed for their amino acid compositions and sequences in the same way.

**CD Analysis**—The formation of the partially foldd I(SH) from the D(SH) state was monitored by CD ellipticity as described before (Hirose and Yamashita, 1991). In brief, the denatured, reduced half-molecule was prepared in the same way as described above, except that the disulfide reduction was done at 12.0 mg/ml protein. The denatured, reduced protein was diluted 200-fold at 6 °C with Buffer A containing 0.5 mM DTT, and the time course of the change in CD ellipticity at 222 nm was recorded at the same temperature with a Jasco J-501C spectropolarimeter using a 0.2-cm cuvette.

**RESULTS**

**Regeneration of Disulfide Bonds with DTT**—A linear disulfide, such as GSSG, is known to form a stable mixed
tein disulfide with protein sulphydryls, which may complicate protein disulfide analyses. Thus, we examined the conditions for efficient oxidative refolding of the half-molecule using a cyclic oxidant, DTT\textsubscript{ox}, which does not form a stable protein-mixed disulfide (Creighton, 1986). Our previous conditions for effective oxidative refolding of ovotransferrin include a two-step procedure in which the denatured, reduced protein is preincubated at a low temperature in the presence of GSH and then reoxidized after addition of a small volume of a high concentration of GSSG as diagrammed in Scheme A. This two-step procedure is, however, practically difficult to carry out when the linear disulfide is replaced by DTT\textsubscript{ox}. Since DTT\textsubscript{red} is a potent reducing agent, an almost insolubly high concentration of DTT\textsubscript{ox} must be present during the second step of oxidative refolding if DTT\textsubscript{red} is added in the first-step incubation. Thus, we searched for one-step conditions with DTT\textsubscript{ox} that are compatible with the two-step conditions with the linear disulfide.

We have shown that the disulfide-reduced form of the N-terminal half-molecule of ovotransferrin assumes a metastable state, I(SH), at 6 °C displaying an intermediate conformation between the native, disulfide-bonded form, N(SS), and the fully denatured, reduced form, D(SH), as determined by the far UV CD spectrum. We examined the rate of the formation of I(SH) from D(SH) by diluting the denatured, reduced protein at 6 °C with Buffer A containing 0.5 mM DTT\textsubscript{red}. The increase in the absolute value of CD ellipticity at 222 nm as a measure of the formation of I(SH) was found to be completed during the period (about 5 s) of manual mixing (data not shown). In contrast, the rate of reoxidation appeared much slower than that of I(SH) formation as shown in Fig. 2A. When the denatured, reduced protein was diluted at 6 °C with 100 volumes of Buffer A containing 100 mM DTT\textsubscript{ox} and reoxidized at the same temperature for various times, it took 2.5 min for about half of the reduced molecule to form a first intrachain disulfide bond in the presence of 100 mM DTT\textsubscript{ox}. Thus, the protein in the D(SH) state should be transformed into the I(SH) state before a first disulfide bond is formed, as long as the one-step procedure is done at 6 °C in the presence of 100 mM DTT\textsubscript{ox}.

Fig. 2B shows that the regenerated number of disulfide bonds after 22 h of reoxidation depends on the concentration of DTT\textsubscript{ox} and that most, if not all, of the half-molecules completed their disulfide bond formation at an oxidant concentration as high as 100 mM.

**Reverse-phase HPLC of Tryptic Digest and Identification of Disulfide Peptides**—To establish the peptide mapping for the native disulfide bonds, the native half-molecule was extensively digested with trypsin, and the tryptic digest was fractionated by reverse-phase HPLC as shown in Fig. 3A. All the peaks were assayed for disulfides by a fluorescence method using SBD-F, and nine disulfide peaks denoted A to I were detected. All nine peaks were further fractionated by rechromatography on the same HPLC column using a different buffer system. In the peaks A, B, D, E, F, G, and H, only one disulfide peptide was detected. However, the peaks C and I

![Fig. 2. Regeneration of disulfide bonds by the reoxidation with DTT\textsubscript{ox}. In panel A, the denatured, reduced protein was reoxidized at 0.02 mg/ml in Buffer A at 6 °C with 100 mM DTT\textsubscript{ox} for different times: lane 2, 0 min; lane 3, 2.5 min; lane 4, 5 min; lane 6, 10 min; lane 7, 20 min; lane 8, 1 h; lane 9, 3 h; lane 10, 5 h; lane 11, 22 h. In panel B, the reoxidation was performed at the same temperature for 22 h with various concentrations of DTT\textsubscript{ox}: lane 2, 12.5 mM; lane 3, 25 mM; lane 4, 50 mM; lane 5, 100 mM. The reoxidized protein was trapped by alkylation with iodoacetic acid, washed by precipitation in cold acetone, fully reduced by DTT\textsubscript{ox}, alkylated with iodoacetamide, and electrophoresed on an acid-urea gel. In lanes 1 and 12 in panel A and lanes 1 and 6 in panel B, the standard protein with different numbers of disulfide bonds was electrophoresed. The numbers on the right side represent the numbers of disulfide bonds in a protein molecule.**

![Fig. 3. Fractionation of the tryptic digests of the native and reoxidized proteins. The native half-molecule (panel A) and the protein reoxidized with 100 mM DTT\textsubscript{ox} for 22 h (panel B) were extensively digested with trypsin, and the tryptic digests were applied to a reverse-phase HPLC column equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted with a linear 0–60% gradient of acetonitrile in 0.1% trifluoroacetic acid and assayed for their disulfides using a fluorescent reagent. Disulfide-positive peaks are denoted by A to I in panel A, and by A to I and X to Z in panel B.](image-url)
were separated into two disulfide peptides, respectively, designated C-1 and C-2 and I-1 and I-2. The purified disulfide peptides were analyzed for their amino acid sequences from N-terminal to C-terminal with a sequenator. All the peptides consisted of two amino acid sequences in almost equimolar amounts. In addition, Table I shows that the occurrence of two half-cystine residues in all the peptides was confirmed by the amino acid analysis. In light of the established sequence and the disulfide bonds of the native ovotransferrin (Williams et al., 1982), the 11 disulfide peptides were assigned as summarized in Fig. 4. As shown in Table I, the amino acid compositions of the disulfide peptides were consistent with the sequence analysis data within the range of experimental error. Fig. 4 shows that from SS-I, SS-IV, or SS-V, a single disulfide peptide was produced by cleavage at a trypsin-specific site. In contrast, multiple cleavage was observed with SS-III and SS-VI. This was due to partial cleavage at a trypsin site or to the possible presence of chymotrypsin activity in the trypsin preparation. The disulfide peptides, C-1 and D, are both generated by cleavage at a trypsin-specific site. This divergence can be accounted for by the microheterogeneity in the primary structure (Ala or Ser at position 33). This microheterogeneity was consistent with the previous observation by Williams et al. (1982).

The disulfide localization in the reoxidized N-terminal half-molecule of ovotransferrin was determined based on the peptide mapping data of the native protein. The denatured, reduced protein was reoxidized by a one-step procedure with DTTa or by a two-step procedure with GSSG. The reoxidized proteins were digested with trypsin, and the digests were fractionated in the same way. Fig. 3B shows that the peptide elution profile of the DTTa-reoxidized protein was very similar to that of the native protein and that all nine peaks, A to I, were detected with the reoxidized protein. A very similar elution profile and the presence of the nine disulfide peaks were also observed with the GSSG-reoxidized protein (data not shown). The nine disulfide peaks were further purified by rechromatography, and the presence of all 11 disulfide peptides was confirmed with either the DTTa- or GSSG-reoxidized protein by the analyses of amino acid sequences and compositions. These data led us to conclude that all six native disulfide bonds are qualitatively regained in the half-molecule reoxidized with either DTTa or GSSG.

**Recovery of Native Disulfide Peptides**—To investigate the reoxidation problem more quantitatively, we determined the recoveries of the disulfide peptides. The entire cycle of the experiments including the reoxidation, the proteolysis of reoxidized proteins, and the purification of the disulfide peptides was done in duplicate with both the reoxidized and native proteins. The recoveries of the disulfide peptides from the pretrypsinized proteins were determined by amino acid analysis on a molar basis, and the recoveries of the protein disulfide bonds were evaluated on the basis of the corresponding peptide recoveries. When disulfide peptides were diverged from a

### Table I

| Amino acids | Molar ratios of amino acids*
|-------------|---------------------------|
|             | A  | B  | C-1 | C-2 | D  | E  | F  | G  | H  | I-1 | I-2 |
| Asp         | 2.15 | 2.28 | 2.24 | 1.20 | 7.80 | 6.02 | 1.27 |
| Ser         | 1.04 | 1.67 | 0.98 | 2.72 | 1.82 | 3.10 | 1.06 | 0.85 | 2.05 |
| Glu         | 1.21 | 1.31 | 1.31 | 2.93 | 4.75 | 4.24 | 2.17 |
| Thr         | 0.96 | 1.04 | 1.92 | 1.03 | 2.90 | 0.95 | 1.83 | 2.52 | 2.68 | 1.80 |
| Gly         | 1.06 | 2.63 | 3.66 | 1.00 | 4.13 | 1.11 | 1.13 |
| His         | 1.62 | 1.75 | 1.74 | 0.87 | 0.83 |
| Ala         | 0.87 | 0.75 | 0.65 | 0.70 | 0.96 | 1.94 | 1.92 | 1.77 | 1.70 | 0.81 |
| Arg         | 2.00 | 2.00 | 1.00 | 1.00 | 1.00 | 2.00 | 2.00 |
| Tyr         | 1.12 | 1.88 | 1.30 | 0.77 | 0.98 |
| Pro         | 1.05 | 1.05 | 0.94 | 1.76 | 1.00 |
| Trp         | 1.05 | 0.82 | 1.01 | 1.78 | 1.02 |
| Phe         | 1.03 | 0.93 | 0.98 | 0.86 | 1.70 |
| Ile         | 2.23 | 1.10 | 2.20 | 2.26 | 2.00 | 1.25 | 2.00 | 1.18 | 2.00 |
| Lys         | 1.07 | 2.15 | 2.06 | 2.23 | 2.07 | 1.96 | 2.77 | 2.75 | 1.08 |
| Cysb        | 1.69 | 1.67 | 2.00 | 1.75 | 1.64 | 1.91 | 1.85 | 1.90 |

* Calculated by normalizing to a value of 1.0 (peptides, C-1, C-2, D, E, G) or 2.0 (peptides, A, B, H, and I-1) for arginine, if it occurs, otherwise, to a value of 2.00 for lysine (peptides, F, and I-2). Data are the averages of duplicate determinations. Numbers in the parentheses represent theoretical values calculated from the sequence data.

b Determined separately as cysteic acid after performic acid oxidation.
Parentheses in Table 1 show that the recoveries of peptide purification, and their chemical analyses was done in duplicates. Parentheses represent the relative recovery of the reoxidized proteins. Disulfide bonds were recovered in significantly good yields reoxidized, and GSSG-reoxidized proteins, respectively. Calculated by the equation: (disulfide recovery from the reoxidized protein)/x100%/(disulfide recovery from the native protein). In contrast, with the native disulfide peptides. The occurrence of two half-cystine residues, which cannot be identified in the sequence analysis, was confirmed by the amino acid analysis as shown in Table 1.

**TABLE II**

Recoveries of the native disulfide peptides

The entire cycle of the experiment including protein reoxidation, peptide purification, and their chemical analyses was done in duplicate, and peptide recoveries were determined by the amino acid analysis. If disulfide peptides were diverged from a protein disulfide bond, their recoveries were summed. Data are expressed as the averages of two experiments.

| Protein disulfide bond | Peptides | Recoveries* of disulfide bonds from N-OVT | R-OVT<sub>prot</sub> | R-OVT<sub>gssg</sub> |
|------------------------|----------|------------------------------------------|---------------------|---------------------|
| I                      | I-2      | 72.0                                      | 70.5 (98)           | 70.8 (98)           |
| II                     | C-1 + D  | 59.0                                      | 63.2 (107)          | 59.7 (101)          |
| III                    | C-2 + E + G | 71.1                                      | 74.6 (105)          | 73.3 (103)          |
| IV                     | F        | 64.5                                      | 32.3 (50)           | 43.2 (67)           |
| V                      | A        | 71.3                                      | 35.0 (49)           | 37.2 (52)           |
| VI                     | B + H + I-1 | 71.5                                      | 72.9 (102)          | 74.1 (104)          |

* Molar recoveries from the pretrypsinized proteins. In the parentheses represent the relative recovery of the reoxidized proteins calculated by the equation: (disulfide recovery from the reoxidized protein)/x100%/(disulfide recovery from the native protein).

**FIG. 4.** Amino acid sequences of native disulfide peptides. The disulfide-positive peaks of A to F in Fig. 3A were further purified by rechromatography, as described in the text, and analyzed for their amino acid sequences by a gas-phase sequenator. The presence of two half-cystine residues, which cannot be identified in the sequence analysis, was confirmed by the amino acid analysis as shown in Table 1.

**FIG. 5.** Amino acid sequences of nonnative disulfide peptides from the reoxidized protein. The disulfide-positive peaks, X, Y, and Z in Fig. 3B, were further purified by rechromatography as described in the text and analyzed for their amino acid sequences by a gas-phase sequenator. The occurrence of two half-cystine residues, which cannot be identified in the sequence analysis, was confirmed by the amino acid analysis after performic oxidation as described in the text. *Q represents possible pyrrolidone ring formation in the N-terminal glutamine.
occurrence of non-disulfide-bonded sulfhydryls in SS-IV and SS-V can be postulated as an alternative possibility for the selectively lower recoveries in the GSSG-reoxidized protein. If SS-IV and SS-V are less stable disulfide bonds than the other four disulfides, the two protein disulfides may be involved in sulfhydryl-disulfide equilibrium. To test this possibility, we have reduced the native half-molecule with various concentrations of DTT_{red} under the same buffer and temperature conditions as in the oxidative refolding and analyzed for the reduced number of disulfide bonds by the PAGE technique. As shown in Fig. 6, two disulfide bonds were the first ones cleaved in the presence of 5–20 mM DTT_{red}; little five disulfide species were detected under all test conditions.

To identify the cleaved disulfide bonds, intact disulfide bonds were determined by making use of the peptide mapping established in Fig. 3. The native protein was reduced for 30 min with 10 mM DTT, alkylated with iodoacetic acid, and then fractionated by reverse-phase HPLC. As shown in Fig. 7A, the partially reduced protein was separated into two major peaks, a and b. Both the peaks, a and b, were further purified by rechromatography using the same column and solvent system. Fig. 7B shows that peaks a and b corresponded to a six disulfide species and a four disulfide species, respectively.

The purified four disulfide protein along with the native protein control was extensively digested with trypsin and fractionated by reverse-phase HPLC in the same way as in Fig. 3. All the peptide peaks were examined by disulfide assay using a fluorescent reagent. As shown in Fig. 8, peaks A and F, which are both nondiverged peptides from the two inner disulfides of the kringle, were missing in the four disulfide protein, but all the other disulfide peaks, B, C, D, E, G, H, and I, were detected. The disulfide-positive peaks were further purified by rechromatography in the same way as in Fig. 3, reconfirmed for their amino acid sequences, and analyzed for amino acid compositions and recoveries. All the disulfide peptides other than A and F were recovered in almost exactly the same yields as the native protein. The recoveries from the four disulfide species relative to those from native protein were 101% for SS-I (peptide I-2), 93% for SS-II (peptides C-1 + D), 86% for SS-III (peptides C-2 + E + G), and 101% for SS-VI (peptides B + H + I-1). Furthermore, new disulfide-negative peaks denoted Q and R, and also peaks with increased intensities compared with the native control, P and S, (refer to Fig. 3A) were detected in the four disulfide protein. The four disulfide-negative peaks were purified by rechromatography and analyzed for their amino acid sequences. The amino acid sequences of the peptides P, Q, R, and S were QXK, XAR, LXR, and FFSASXPGATIEQK, respectively. The amino acid analysis revealed that the unknown amino acid, X, corresponds to carboxymethylcysteine. These data indicated that the peptides P and S correspond to the reduced and alkylated peptides from the disulfide peptide F and the peptides Q and R to those from the disulfide peptide A. Considering these facts, we concluded that SS-IV and SS-V are selectively cleaved by reduction with 10 mM DTT_{red}.

**DISCUSSION**

The data in the present study demonstrate that all six native disulfide bonds are regained in the reoxidized N-terminal half-molecule of ovotransferrin. To our knowledge, this is the first demonstration of the regaining of native disulfide bonds in an oxidatively refolded form of a multidomain protein with many disulfides. We have previously shown that the disulfide-reduced half-molecule assumes at a low temperature a unique molten globule-like state, I(SH), that can be differentiated from either the denatured, disulfide-reduced form, D(SH), or the native, disulfide-bonded form, N(SS), and that
proteins, because BPTI (Kosen et al., 1992) is employed with the circular disulfide, D(SH), as the immediate precursor of their disulfide-reduced state. I(SH) is much more rapid than that of the reoxidation of a molten globule-like state of I(SH) is involved as long as low temperature conditions are employed in the oxidative folding pathway of the half-molecule as the immediate precursor of the disulfide-reduced ovotransferrin is in great contrast to the oxidative refolding pathway of small disulfide proteins, because BPTI (Kosen et al., 1983), lysozyme (Acharya and Tanemichi, 1982), and ribonuclease A (Galat et al., 1981) essentially assume an unfolded conformation in their disulfide-reduced state.

BPTI has been shown to produce a mixture of two species on apparent completion of oxidative refolding. One is a native form, and the other is a nonproductive intermediate, designated N*, which lacks one of the three native disulfide bonds (States et al., 1984). Similarly, the recoveries of SS-IV and SS-V from the reoxidized proteins were about 50% of those from the native protein, but the recoveries of SS-I, SS-II, SS-III, and SS-VI were almost equivalent between the native and reoxidized proteins. The two sulfhydryls in the nonproductive species of BPTI are buried in the interior of the protein molecule, which restricts access by an alkylation or oxidation reagent. Similar inaccessible sulfhydryls are also observed during the oxidative refolding of the constant fragment of immunoglobulin light chain (Goto and Hamaguchi, 1981). This inaccessible model, however, is very unlikely for sulfhydryls of SS-IV and SS-V, because the four sulfhydryls from these disulfides are all reactive with monoiodoacetic acid (Fig. 6). Data in the present paper are consistent with an alternative mechanism in which the four disulfide species lacking SS-IV and SS-V is involved as a productive intermediate.

The two disulfide bonds, SS-IV and SS-V, were the first ones cleaved with DTTrred under the same buffer and temperature conditions as those of oxidative refolding (Figs. 6-8). Creighton (1978) has shown that the kinetics of disulfide reduction in BPTI is consistent with the unfolding pathway being the reverse of the folding pathway. If the disulfide reduction of SS-IV and SS-V corresponds to the reverse reaction of the oxidative refolding, Scheme A for the oxidative refolding process of ovotransferrin can be revised as a more specific form for the N-terminal half-molecule

\[ D[2SH] \rightarrow I[2SH] \rightarrow \cdots \rightarrow I'[4SH/4SS(II,III,VI)] \]

\[ \text{N[6SS(II-VI)]} \]

Scheme B

where I'[4SH/4SS(II,III,VI)] represents the four disulfide-bonded species lacking the two inner disulfide bonds in the kringle. GSH is present in the reoxidation system using glutathione. Thus, the selectively lower recoveries of SS-IV and SS-V may be explained at least in part by the involvement of a protein sulfhydryl and disulfide equilibrium during the last stage of reoxidation using the glutathione system.

As another observation related to the four disulfide-bonded species, significant amounts of nonnative disulfide bonds, Cys<sup>171</sup>-Cys<sup>174</sup> and Cys<sup>174</sup>-Cys<sup>182</sup>, were detected in the protein reoxidized with DTTrred. These nonnative disulfides might be accounted for by incorrect disulfide formation in the four disulfide intermediate lacking SS-IV and SS-V. In the oxidative refolding of BPTI, nonnative two disulfide species are encountered as transient intermediates involved in disulfide rearrangement (Creighton and Goldenberg, 1984; Weissman and Kim, 1991, 1992). The nonnative intermediate of BPTI is probably significantly unfolded, because incorrectly paired sulfhydryls are quite far from each other in the native conformation. The crystallographic data<sup>1</sup> for the N-terminal half-molecule of ovotransferrin are, however, inconsistent with such a greatly perturbed conformation for the half-molecule having the nonnative disulfides. Srinivasan et al. (1990) have shown that the C<sub>j</sub>-C<sub>j</sub> distances of 72 disulfide bonds in 22 proteins occur in a narrow range of 3.5-4.5 Å. The C<sub>j</sub>-C<sub>j</sub> distances of the two native cystine disulfides (SS-IV and SS-V) are indeed within the range. The C<sub>j</sub>-C<sub>j</sub> distances between Cys<sup>171</sup> and Cys<sup>174</sup> and between Cys<sup>174</sup> and Cys<sup>182</sup> are only about 5 Å in the native conformation. It is, therefore, very unlikely that the generation of the nonnative disulfide bonds greatly perturbs the native conformation of ovotransferrin. In our putative model, the three-dimensional localization of the C<sub>j</sub> atoms of the 4 cysteine residues in SS-IV and SS-V is very similar in the native, four-disulfide, and nonnative disulfide proteins. This may result in the acquisition of a similar disulfide stability by the native and nonnative disulfide bonds and also in a lower disulfide stability in SS-IV and SS-V.

In conclusion, we believe that the present data for the

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<sup>1</sup> B. Mikami, J. Dewan, J. Sacchettini, and M. Hirose, oral presentation at the annual meeting of Japan Society for Bioscience, Biotechnology, and Agrochemistry (March, 1993, Sendai).
chemical structure of the reoxidized N-terminal half-molecule of ovotransferrin provide an important basis for investigating the folding mechanism of multidomain proteins. A detailed pathway for reversible unfolding will be established by the disulfide analyses of all the intermediate species involved in the oxidative refolding and reductive unfolding processes of the N-terminal half-molecule of ovotransferrin.

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