STUDIES ON THE FOLDING PROCESSES
OF EGG WHITE PROTEINS

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CONTENTS

Abbreviations

CD	circular dichloism
DTNB	5,5'-dithiobis (2-nitrobenzoate)
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetate
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IAA	iodoacetic acid
IAEDANS	5-[2-[[iodoacety]amino]ethyl]amino aminonaphthalen-1-sulfonic acid
IAM	iodoacetamide
PAGE	polyacrylamide gel electrdphoresis
SDS	sodium dodecyl sulfate
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)-aminomethane

INTRODUCTION

The conformation of a protein has been believed to be determined solely from its amino acid sequence. This was stated since the finding of the recovery of ribonuclease A activity by oxidation of the fully reduced and unfolded molecule by Anfinsen (1). The folding of a polypeptide chain has been thought to reach a distinct native three dimensional structure in a spontaneous way. Actually, most purified proteins spontaneously refolded in vitro after being completely unfolded. This has been, however, restricted on the relatively small and simple proteins such as bovine pancreatic trypsin inhibitor, lysozyme, ribonucleases, and the constant fragment of immunoglobulin light chain (2-5). Any complex protein which has several disulfide bonds or large molecular size has never regain its native conformation after denaturation and full reduction. On the production of the recombinant proteins in host microorganisms, such complex proteins tend to form "inclusion-bodies" as inactive deposits (6). Since even such a protein, however, gets the correct native structure in the cell in which the protein has been intrinsically expressed, intracellular environment or any factor in a living cell can be required to the folding of the complex proteins (7). In the light of the cell biology, several abundant, cellular proteins which related to the completion of

the protein folding have been found out (protein disulfide isomerase, proryl cys-trans isomerase, heat shock related proteins, etc)(8-11). However, these factors are only thought to avoid falling into some disadvantages such as incorrect disulfide bonds, non-native proline isomers, and coagulation mediated by hydrophobic interaction.

In this study, the author attempted to achieve complete recovery of the conformation of two relatively complex egg white proteins. Through this process, misfolded intermediates were unavoidable and they could not be distinguished by any conventional methods. Thus, a highly-specific and sensitive method for determination of sulfhydryls and disulfides was developed in chapter 1. Using this technique, an optimal procedure was searched for the refolding of ovotransferrin as a model of protein with many disulfide bonds in chapter 2. As a complex protein which has both disulfide bond and several free sulfhydryls, ovalbumin was examined on its refolding process in chapter 3.

CHAPTER 1

A method for determination of sulfhydryl groups
and disulfide bonds by polyacrylamide gel electrophoresis

The cysteine sulfhydryl and cystine disulfide have functional, structural and biological significance in protein molecules. The cysteine sulfhydryl group is essential for the catalytic activity of some enzymes (12) ; the interconversion between sulfhydryls and a disulfide is directly involved in the activity of thioredoxin and protein disulfide isomerase (13). Intramolecular disulfide bonds are involved in the stabilization of the folded conformation of many proteins (14). Cellular disulfide bond formation is an important process in the post-translational modification of secretory proteins (15). The conventional methods for the quantitative analysis of protein sulfhydryls and disulfides are the amino acid analysis and spectrophotometric methods (16). These methods, however, are not suitable for the analysis in biological systems, where only a small amount of a protein in question is contained in lots of non-specific proteins. Furthermore, several intermediates that have different number of disulfide bonds has not been distinguished by the conventional methods. Such intermediates can be generated both in the biological systems and in the in vitro refolding process.

In the aim for understanding of the protein folding, an alternative method which is able to differentiate the many intermediates and is applicable to biological systems has been required. PAGE method is a strong candidate because of its high sensitivity, and highly specific staining.

Creighton has first developed a method by PAGE to determine total half-cystines per protein molecule (17). The method includes the complete cleavage of disulfide bonds by a thiol, alkylation with varying ratios of IAA to IAM, and low-pH urea PAGE. The integral number of total half-cystines per protein molecule can be calculated from the number of the protein bands. This depends on the generation of a complete set of protein species having 0, 1, 2,..., n acidic IAA-carboxyls, where n is the total number of half-cystines per protein molecule.

In this chapter, an alkylation method which can be analyzed by specific and sensitive PAGE has been developed, so that all the half-cystine residues in a native protein can be categorized into these three states: (i) disulfide bonded; (ii) reactive sulfhydryls; (iii) nonreactive sulfhydryls. As diagrammed in Figure 1, protein sulfhydryls have been alkylated in the first step with IAA in the presence and absence of a high concentration of urea; in the second step, disulfide bonded ones have been fully reduced and alkylated with IAM. We show that the use of high-pH urea PAGE has enabled us to determine the states of half-
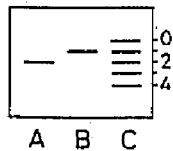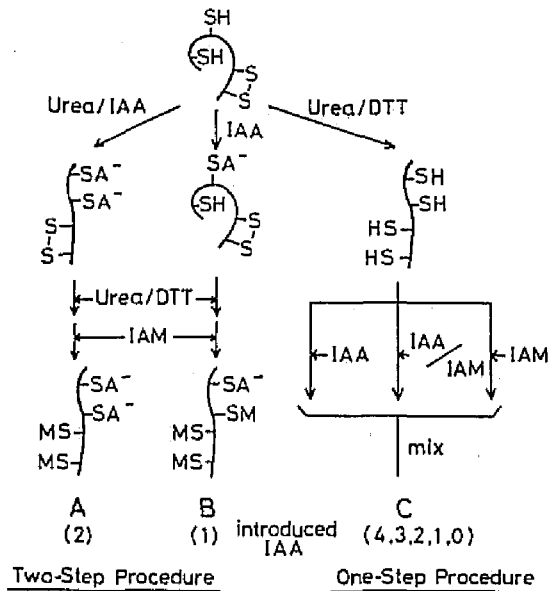
Fig. 1. Strategy for the determination of sulfhydryls and disulfides in a protein. A hypothetical protein has one intramolecular disulfide bond, one reactive sulfhydryl group and one nonreactive sulfhydryl in its native conformation.

In the one-step alkylation procedure, the protein is fully reduced and alkylated with varying molar ratios of IAA to IAM, five protein species with different introduced number of IAA carboxyls (0, 1, 2, 3, and 4) should be generated. If the five species are distinctly separated on a denaturing polyacrylamide gel, we can use the migration pattern as a standard for monitoring the relation between protein mobilities and introduced number of IAA carboxyls, as in lane C.

In the two-step procedure, the first-step alkylation is performed in the presence and absence of a high concentration of urea: one reactive sulfhydryl and two sulfhydryls that correspond to the sum of one reactive and one nonreactive sulfhydryls should be modified with IAA in the absence and presence of urea, respectively. The protein is fully reduced and alkylated with IAM in the second step. On the same polyacrylamide gel, introduced number of IAA carboxyls can be determined, as in lane A and B. Nonreactive two half-cystines in the presence of urea in the first step should be accounted for by one disulfide.

cystine residues in several types of proteins having sulfhydryls and/or disulfides. The particular advantages of the method are that the state of half-cystines in different protein species can be determined independently both in isolated proteins and de novo translation products. These are demonstrated with a model protein system of ovalbumin that contains four sulfhydryls and one intramolecular disulfide. Other egg white proteins, lysozyme with 4 disulfide bonds and ovotransferrin with 30 disulfide bonds are investigated for the disulfide formation in isolated tissue explants.

MATERIALS AND METHODS

Materials

The N-terminal and C-terminal half-molecules of ovotransferrin were prepared as described before (18). Dephosphorylated ovalbumin prepared by the treatments with acid phosphatase and ion-exchange chromatography (19) was generously given by Dr. N. Kitabatake (Kyoto University). E. coli thioredoxin (rProFold) was obtained from Takara Biochemicals. Soy bean trypsin inhibitor, porcine pancreatic phospholipase A_2 and hen egg white lysozyme were purchased from Sigma. Oligo(dT)-cellulose was purchased from Collaborative Research Inc. L-[^{35}S]Methionine (1,230 Ci/mmol) and methionine-depleted

amino acid mixture were obtained from Amersham. Emetin hydrochloride was purchased from Tokyo Kasei. The native, reduced form of thioredoxin was prepared by the incubation of the commercially obtained thioredoxin with 10 mM DTT at 37°C for 30 min in Buffer A (50 mM Tris pH 8.2/1 mM Na$_2$EDTA). DTT was removed by gel filtration using a Sephadex G-25 column (Pharmacia LKB Biothchnology, NAP-5) equilibrated with the same buffer. Alkylated lysozyme used as a carrier for protein precipitation by acetone was prepared by alkylation with IAM in Buffer A containing 8 M urea at 37°C for 30 min and excess IAM was removed by gel filtlation with the prepacked Sephadex G-25 column.

One-step alkylation procedure

One step alkylation was performed essentially as described by Creighton (17). A protein with intramolecular disulfides was fully reduced at 0.2 mg/ml by the incubation with 3.5 mM DTT at 37°C for 30 min in 0.5 ml of Buffer A containing 8 M urea. The sample was subdivided into five portions. They were alkylated at 37°C for 10 min by different molar ratios of IAA to IAM (IAA/IAM: 30 mM/0 mM; 22.5 mM/2.5 mM; 15 mM/5 mM; 7.5 mM/7.5 mM; 0 mM/10 mM), mixed, and used for PAGE. In some experiments, the protein alkylated with either IAA or IAM alone was electrophoresed without mixing. When the mixed sample was needed to be stored, free IAA and IAM were removed by passing the

sample through a Sephadex G-25 column (NAP-25, Pharmacia) equilibrated with 50 mM Tris-HCl buffer, pH 8.2 containing 6 M urea, 10% glycerol and 1 mM Na_2EDTA.

Two step alkylation procedure

In the first step, a protein was alkylated at 0.2 mg/ml with 30 mM IAA at $37^O C$ for 15 min in 0.5 ml of Buffer A in the presence and absence of 8 M urea. To the mixture, 0.01 volume of the carrier protein (5 mg/ml of alkylated lysozyme) and 10 volume of cold acetone/1N-HCl (98:2) were added, and then proteins were precipitated by centrifugation (3,000 x g, 5min). After precipitates were washed three times by repeated resuspension in cold acetone/1N-HCl/H_2O (98:2:10) and centrifugation (3,000 x g, 5 min), proteins were dissolved in 0.5 ml of Buffer A containing 8 M urea, fully reduced by an incubation with 3.5 mM DTT at $37^O C$ for 30 min, and alkylated with 10 mM IAM at $37^O C$ for 10 min in the second step.

Denaturing polyacrylamide gel electrophoresis

Proteins were electrophoresed on a discontinuous acrylamide slab gel (13.5 x 13.5 x 0.1 cm) in a high-pH buffer system described by Davis (20). Stacking and resolving gels contained 0.12 M Tris-HCl buffer, pH 6.8 and 0.037 M Tris-HCl buffer, pH 8.8, respectively. Reservoir buffer consisted of 0.025 M

Tris-0.192 M glycine, pH 8.3.   The concentration of acrylamide
was 9 % in the separating gel and 2.5 % in the stacking gel.
Both the stacking and separating gels contained 8 M urea.
Electrophoresis was performed at $4^{O}C$ for 15 h at a constant
current of 10 mA (ovalbumin and the half molecules of
ovotransferrin) or 5 mA (soy bean trypsin inhibitor,
phospholipase A_2 and thioredoxin).   Proteins were stained with
Coomassie blue.   Low-pH urea PAGE was carried out in the
presence of 8 M urea and the buffer system described by Reisfeld
et al..   The concentration of acrylamide in the separating gel
was 7.5 % for the analysis of ovotransferrin and its half-
molecules, and 15 % for lysozyme.   Proteins were run at constant
currents (10-20 mA) for 16-22 hr at 8 ^{O}C and were stained with
coomassie blue.

Spectrophotometric determinations of sulfhydryl groups

    Ovalbumin (0.2 mg/ml) was incubated at $37^{O}C$ for 15 min in
Buffer A containing various concentrations of urea.   To the
sample, 0.01 volume of 10 mM DTNB was added.   After 10 min at
room temperature, absorbance at 412 nm was measured, and reactive
sulfhydryls were determined on the basis of molar extinction
coefficient of 13,600 $M^{-1}cm^{-1}$ (21).

Analysis of a cell-free translation product

Total RNA was extracted from laying hen oviducts, and poly-A$^+$RNA fraction was obtained by oligo(dT)-cellulose column chromatography (22). Wheat germ extracts utilized for cell-free translation were prepared as described elsewhere (23). The reaction mixture consisted of 26 mM Hepes, pH7.6, 2 mM DTT, methionine-depleted amino acid mixture (50 μ M each), 0.75 mM magnesium acetate, 43 mM potassium acetate, 125 μ M spermine, 1 mM ATP, 250 μ M GTP, 20 mM phosphocreatine, 0.5 μ g/ml phosphocreatine kinase, 0.1 mg/ml poly-A$^+$RNA, 0.3 mCi/ml [^{35}S]methionine, 26.2 A$_{260}$ units/ml of wheat germ extract in the presence or absence of varying concentrations of oxidized glutathione in a final volume of 20 μ l. After 60 minute-reaction at 25°C, translation was terminated by the addition of 0.01 volume of 11 mM emetine. For the one-step alkylation, the sample translated in the absence of oxidized glutathione was centrifuged at 4°C for 60 min at 100,000 x g with a ultracentrifuge (Hitachi ultracentrifuge, type 70-P). Proteins in the polysome-depleted supernatant were precipitated in cold acetone/1N-HCl, dissolved in Buffer A containing 8 M urea and 3 mM DTT, incubated at 37°C for 30 min, and then alkylated with the different molar ratios of IAA to IAM as described above. For the determination of disulfide formation, the samples translated in the presence of varying concentrations of oxidized glutathione were incubated with 50 mM IAA for 5 min prior to the ultra-

centrifugation.   Translation products in supernatant were diluted with four volumes of Buffer A containing 10 M urea and 50 mM IAA, incubated at 37°C for 10 min, and then precipitated in the cold acetone/HCl solution.   The precipitate was dissolved in Buffer A containing 8 M urea and 3 mM DTT, incubated at 37°C for 30 min, alkylated with 10 mM IAM at 37°C for 15 min.   The sample was analyzed by the high-pH urea PAGE.   Proteins were detected by fluorography using Kodak X-Omat AR film and ENLIGHTNING (New England Nuclear).

Analysis of Egg White proteins synthesized in isolated chick oviduct explants

Female White Leghorn chicks were stimulated with estrogen for 14 days by subcutaneous implantation of one tube (2.5 cm long, Silastic medical-grade tubing, Dow Corning) containing 65 mg of diethylstilbestrol per chick.   Oviducts were removed, minced into small pieces (about 1 mm^{3}), and washed twice with Buffer B (20 mM Hepes-HCl, pH 7.7, Hank's salt).   The egg white proteins were labeled by incubation of the tissues (0.3 g, wet weight) at 37°C for 15 min in 0.5 ml of Buffer B containing 220 Ci/ml of [^{35}S]methionine.   The tissues were washed twice with cold Buffer B containing 50 mM IAA or IAM, and immediately homogenized in Buffer C (25 mM Tris-HCl, pH 7.5, 25 mM NaCl, 0.25 % Triton X-100, and 0.25 % sodium deoxycholate) containing 50 mM

IAA or IAM as previously described (24). Homogenates were then centrifuged at 12,000 g for 15 min. The first-step alkylation was done by incubation of the supernatant at 37°C for 15 min. The sample was chilled on ice, and free IAA or IAM was removed by passing through a Sephadex G-25 column (NAP-10, Pharmasia) equilibrated with Buffer C.

Ovotransferrin synthesized $\underline{in}$ $\underline{vitro}$ was purified with an immobilized anti-ovotransferrin column (0.8 cm diameter X 0.5 cm long). A portion of the sample was loaded on the column and the bound fraction was eluted with 0.1 N HCl. The eluate was neutralized with Tris-HCl buffer (pH 8.0) at the final concentration of 0.2 M and concentrated with a microconcentrater (Centricon-10, Amicon).

Lysozyme was purified essentially as reported earlier (25). The sample treated with Sephadex G-25 was placed on a Bio-Rex 70 column (0.8 cm diameter X 1.0 cm long) equilibrated with 0.1 M sodium phosphate buffer (pH 8.0). Lysozyme was eluted with 1.0 M of the same buffer and concentrated in the same way. The sample diluted with Buffer A were fully reduced by DTT, alkylated with IAM or IAA in the second step of alkylation, and electrophoresed on low pH urea gel (26). Radiolabeled proteins were made visible by fluorography in the same way.

RESULTS AND DISCUSSION

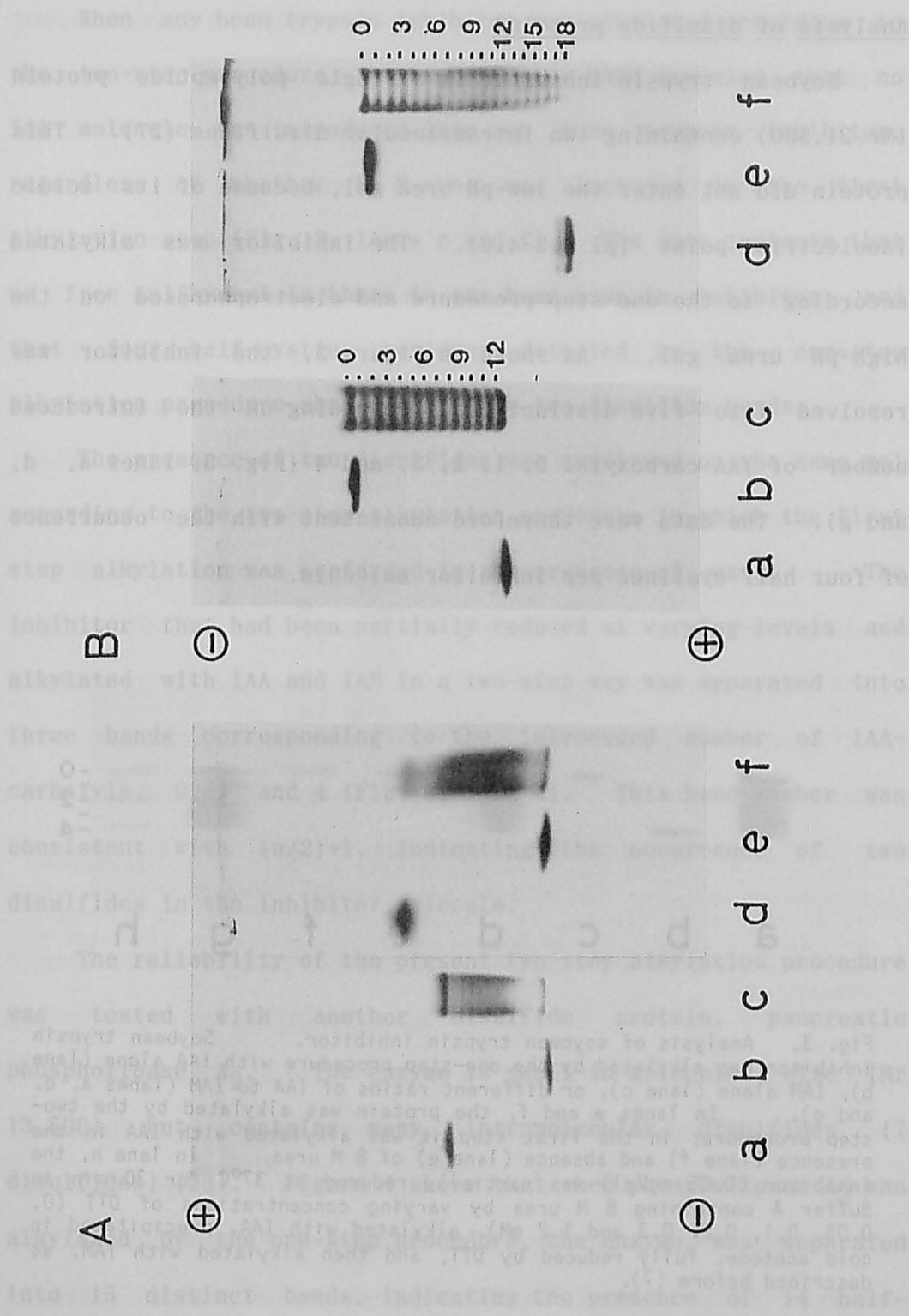### Buffer conditions for discontinuous polyacrylamide gel electrophoresis

A previous report by Creighton has shown that a small disulfide protein (Mr 5,000-12,000 ; 3-4 disulfides), treated with the one-step alkylation procedure, can be separated by a low-pH urea PAGE into n+1 distinct bands, where n is the integral number of total half-cystines. We have shown that by the same low-pH urea PAGE more complex disulfide proteins, the N-terminal (Mr 36,000 ; 6 disulfides) and C-terminal (Mr 38,000 ; 9 disulfides) half molecules of ovotransferrin, can be separated into (n/2)+1 bands, when the proteins are processed for the determination of intramolecular disulfides by a two-step alkylation procedure with IAA and IAM. For the analyses of sulfhydryls and disulfides in variety of proteins according to the present strategy (Fig. 1), PAGE conditions that can distinctly resolve complex proteins as well as simple ones into n+1 bands are prerequisite.

The carboxyl groups of introduced IAA should dissociate more completely at higher pH values. The two half-molecules of ovotransferrin were alkylated by the one-step alkylation procedure, and electrophoresed on polyacrylamide gels under two different buffer conditions. Figure 2 (panel A) shows that the half-molecules with different number of introduced IAA-carboxyls

were poorly resolved in the low-pH urea PAGE. In contrast, the same ovotransferrin samples were distinctly resolved into 13 and 19 bands with respect to the N-terminal and C-terminal half-molecules, respectively, under the high-pH buffer conditions (Fig.2, panel B). These band numbers were consistent with n+1.

Although the low-pH urea PAGE may be still necessary for the analyses of highly basic proteins, including hen egg white lysozyme and pancreatic RNase, the high-pH denaturing PAGE should provide more general conditions for the determinations of total number of half-cystine residues and of the relation between protein mobilities and the introduced number of IAA-carboxyls.

Fig. 2. Effects of buffer conditions on the electrophoretic resolution of ovotransferrin half-molecules. The N-terminal (lanes a to c) and the C-terminal half-molecule (lanes d to f) of ovotransferrin were alkylated by the one-step procedure. The proteins alkylated with IAA alone (lanes a and d) and IAM alone (lanes b and e), and the mixed proteins after the modification with different molar ratios of IAA to IAM (lanes c and f) were electrophoresed on the low-pH urea gel (panel A) and the high-pH urea gel (panel B).

Fig. 1. Analysis of soybean trypsin inhibitor. Soybean trypsin inhibitor was alkylated by the one-step procedure with IAA alone (lane b), IAN alone (lane c), or different ratios of IAA to IAN (lanes d, e, and f). In lanes e and f, the protein was alkylated by the two-step procedure in the presence of ... (lane f) and absence (lane g) of 8 M urea. In lane h, the protein was ... Buffer A contained 8 M urea by varying concentration of DTT.

Analysis of disulfide proteins

Soybean trypsin inhibitor is a single polypeptide protein (Mr 21,500) containing two intramolecular disulfides (27). This protein did not enter the low-pH urea gel, because of its acidic isoelectric point (pI 4.3-4.6). The inhibitor was alkylated according to the one-step procedure and electrophoresed on the high-pH urea gel. As shown in Figure 3, the inhibitor was resolved into five distinct bands depending on the introduced number of IAA-carboxyls: 0, 1, 2, 3, and 4 (Fig. 3, lanes a, d, and g). The data were therefore consistent with the occurrence of four half-cystines per inhibitor molecule.
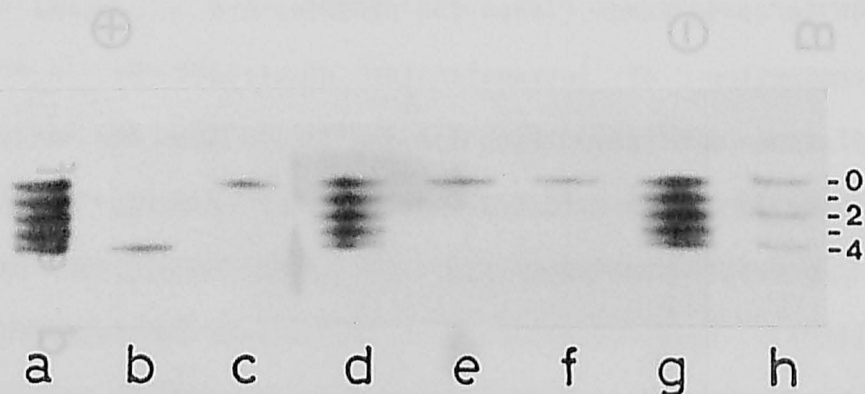

Fig. 3.   Analysis of soybean trypsin inhibitor.   Soybean trypsin inhibitor was alkylated by the one-step procedure with IAA alone (lane b), IAM alone (lane c), or different ratios of IAA to IAM (lanes a, d, and g).   In lanes e and f, the protein was alkylated by the two-step procedure; in the first step it was alkylated with IAA in the presence (lane f) and absence (lane e) of 8 M urea.   In lane h, the inhibitor (0.05 mg/ml) was partially reduced at 37°C for 30 min in Buffer A containing 8 M urea by varying concentrations of DTT (0, 0.05, 0.1, 0.2, 0.3 and 1.2 mM), alkylated with IAA, precipitated in cold acetone, fully reduced by DTT, and then alkylated with IAM, as described before (7).

When soy bean trypsin inhibitor was alkylated according to the two-step procedure, the high-pH urea PAGE revealed that no IAA molecule was introduced into soy bean trypsin inhibitor, regardless of whether 8 M urea was included in the first alkylation step (Fig. 3, lanes e and f). The data indicate that no free sulfhydryl is there in soy bean trypsin inhibitor, and that four half-cystine residues detected by the one-step alkylation procedure should come from two disulfide bonds.

The presence of two disulfides was confirmed on the same gel according to the two-step alkylation procedure in which the first step alkylation was performed in the presence of urea. The inhibitor that had been partially reduced at varying levels and alkylated with IAA and IAM in a two-step way was separated into three bands corresponding to the introduced number of IAA-carboxyls, 0, 2, and 4 (Fig. 3, lane h). This band number was consistent with $(n/2)+1$, indicating the occurrence of two disulfides in the inhibitor molecule.

The reliability of the present two-step alkylation procedure was tested with another disulfide protein, pancreatic phospholipase A_2. The enzyme is small in molecular size (Mr 13,500) but contains many intramolecular disulfides (7 disulfides) (28). Figure 4 shows that when phospholipase A_2 was alkylated by the one-step procedure, the enzyme was separated into 15 distinct bands, indicating the presence of 14 half-

cystines (lanes a and d). No reactive sulfhydryl was detected
in phospholipase A$_2$ both in the presence and absence of 8 M urea
(Fig. 4, lanes b and c). All the 14 half-cystines should be
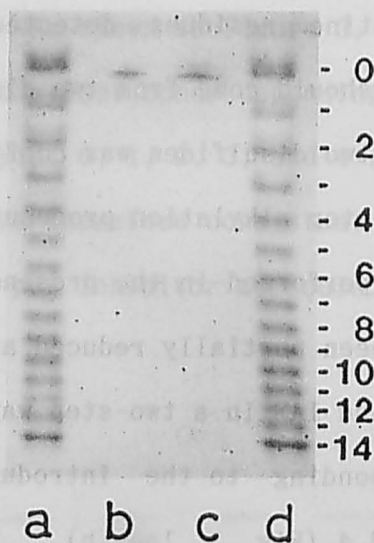therefore covalently bonded with each other.

Fig. 4. Disulfide bonds in phospholipase A$_2$. Pancreatic
phospholipase A$_2$ was alkylated by the one-step procedure with varying
molar ratios of IAA to IAM (lanes a and d) or by the two-step
procedure (b and c). In lanes c and b, the enzyme was alkylated in
the first step with IAA in the presence and absence of 8 M urea,
respectively.

Although both IAA and IAM are known to be a specific reagent

attacking protein sulfhydryls, a histidine residue in hen egg

white lysozyme and a glutamic acid residue in ribonuclease T$_1$ are

reactive to the reagents in their native protein form (29,30).

Such alkylation is to be accounted for by unusually high reactivities of the residues due to their specified location on native protein surfaces. That no IAA is introduced into the native forms of trypsin inhibitor and phospholipase A_2 in the first alkylation step shows the specific modification to sulfhydryls at least with these proteins. No IAA introduction is also observed with the oxidized form of thioredoxin (see forthcoming section). Unusual modification by IAA, if any, could be detected by a modified two-step procedure, where a protein is alkylated with IAA both in the first and second steps.

Another problem is that the used urea conditions are not sufficient for the complete denaturation of a stable protein, leading to an ambiguous conclusion concerning whether nonreactive free sulfhydryls are assigned to disulfide bonded ones. In this case, more drastic denaturation conditions, including temperature and urea concentrations should be employed for the first-step alkylation.

The active site of thioredoxin

Thioredoxin is a biologically important protein (Mr 11,700); its active site, Cys-Gly-Pro-Cys, is directly involved in many cellular redox reactions, thereby the two cysteine residues undergo sulfhydryl to disulfide interchange (31). No other cysteine residue occurs in bacterial thioredoxin (13).
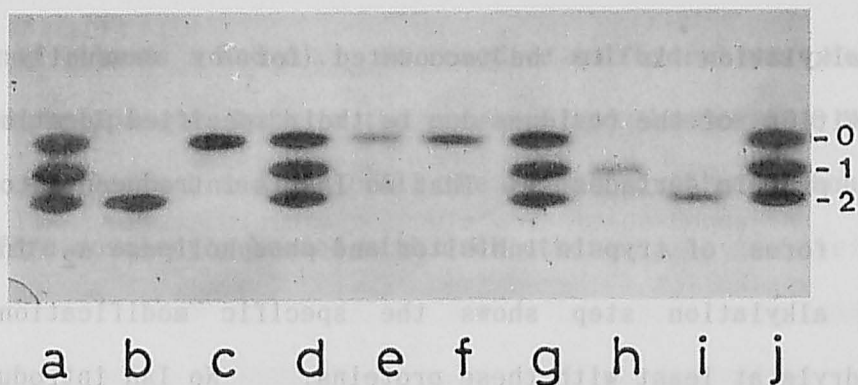
Fig. 5.   Reactivity of sulfhydryl groups in thioredoxin.
Thioredoxin with (lanes h and i) or without (lanes e and f) the
pretreatment of disulfide reduction was alkylated by the two-step
procedure that included the first-step alkylation with IAA in the
presence (lanes f and i) and absence (lanes e and h) of 8 M urea.
The one-step alkylation was carried out with IAA alone (lane b), with
IAM alone (lane c), or with varying ratios of IAA to IAM (lanes a, d,
g, and j).

The protein was alkylated by the one-step procedure, and
electrophoresed on the high-pH urea gel.   Thioredoxin was
distinctly separated into three bands, each corresponding to
introduced number of IAA, 0, 1, and 2, as predicted from the
occurrence of two half-cystine residues (Fig. 5, lanes a, d, g,
and j).

The redox state of the two half-cystine residues was
analyzed by the two-step alkylation procedure.   High-pH urea
PAGE revealed that no IAA was introduced into thioredoxin in the
presence and absence of urea (Fig. 5, lanes e and f).   This was
consistent with the previous data, showing that isolated
thioredoxin takes the oxidized form (32).   In contrast, the
native, reduced thioredoxin was reactive to IAA.   When 8 M urea

was included in the first alkylation step, two IAA molecules were introduced (Fig. 5, lane i); in the absence of urea in the first step, the protein species with one introduced IAA-carboxyl was detected as the major band. The band with two introduced IAA-carboxyls was only trace. Use of higher concentration of IAA (50 mM) in the first step (in the absence of urea) did not resulted in a significant increase in the protein species with two introduced IAA-carboxyls (data not shown). These data agree with the previous report in which Cys-32 with abnormally low pK value, but not Cys-35 with normal pK, can be alkylated by IAA (33).

Reactivity of the sulfhydryl groups of ovalbumin

Ovalbumin consists of a single polypeptide chain (Mr 42,700) that has one disulfide bond and four free sulfhydryl groups (34). In the native state, the four sulfhydryls are not reactive with DTNB (35); but some of them are reactive under denaturing conditions (36). By the present PAGE procedure, we evaluated reactivities of the four sulfhydryls in the presence of varying concentrations of urea.

Dephosphorylated ovalbumin was alkylated according to the one-step procedure, and electrophoresed on the high-pH urea gel. Separation into seven distinct bands (Fig. 6, panel A, lanes a and l) was consistent with the occurrence of six half-cystines in

ovalbumin. In the two-step alkylation, varying concentrations of urea were used in the first step. Dephosphorylated ovalbumin was alkylated with IAA in the presence of 0 to 9 M urea in the first step, and then processed by the second-step procedure in the same way. Four reactive sulfhydryls were detected at urea concentration of 8 M or greater (Fig. 6, panel A, lanes j and k), indicating that all four free cysteine residues are reactive at the high urea concentrations. Nonreactive two half-cystines in the high concentrations of urea should therefore be accounted for by the one disulfide bond. At an urea concentration of 5.5 M or lower, no sulfhydryl was reactive to IAA in the first step (Fig. 6, panel A, lanes b-e). The electrophoretic profile suggested the occurrence of some conformational transition at intermediate urea concentrations of 6 to 7.5 M. The important in this transition was that as typically seen at 6.5 M urea, only two protein species that corresponded to the species with no reactive sulfhydryl and to the species with four reactive sulfhydryls were detected. The protein species having one, two and three reactive sulfhydryls were only trace or almost undetectable (Fig. 6, panel A). The gel was traced with a densitometer, and the band intensities of the two species were determined. The amount of protein species with no reactive sulfhydryl decreased with increasing urea concentration; inversely, protein species with four reactive
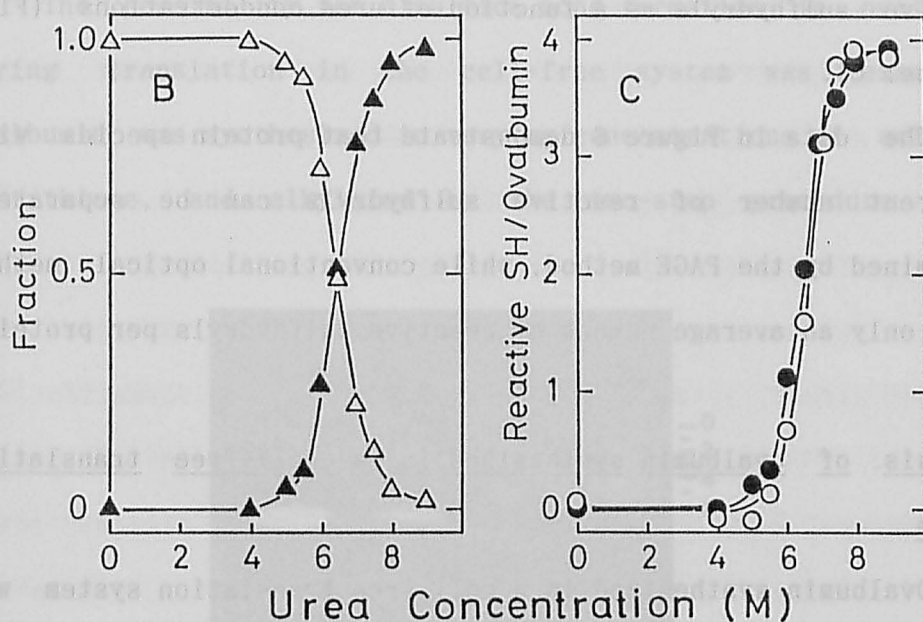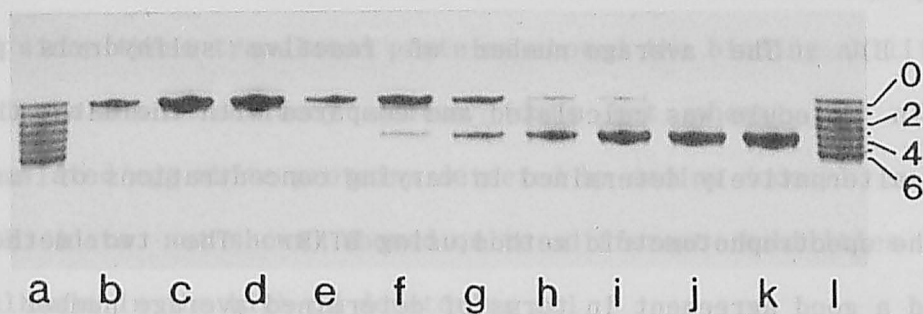
Fig. 6. Effects of urea concentration on the reactivity of sulfhydryl groups of ovalbumin.

(Panel A) In the first step of the two-step procedure, dephosphorylated ovalbumin was alkylated with IAA in the presence of varying concentrations of urea: lane b, 0 M; lane c, 4 M; lane d, 5 M; lane e, 5.5 M; lane f, 6 M; lane g, 6.5 M; lane h, 7 M; lane i, 7.5 M; lane j, 8 M; lane k, 9 M. In lanes, a and l, the protein was alkylated by the one-step procedure with varying ratios of IAA to IAM.

(Panel B) The gel in panel A were traced with a densitometer, and the band intensities corresponding to the protein species with no introduced IAA-carboxyl (△) and those with four introduced IAA-carboxyls (▲) were determined. The ordinate represent the fraction of each protein intensity to the sum of the two species.

(Panel C) The average number of reactive sulfhydryls was determined by the spectrophotometric technique using DTNB (○) or calculated, depending on the data in the panel B, as four times of the fraction of ovalbumin species with four reactive sulfhydryls (●).

sulfhydryls increased with increasing urea concentration (Fig. 6, panel B). The average number of reactive sulfhydryls per protein molecule was calculated and compared with the data that were alternatively determined in varying concentrations of urea by the spectrophotometric method using DTNB. The two methods showed a good agreement in terms of determined average number of reactive sulfhydryls as a function of urea concentrations (Fig. 6, panel C).

The data in Figure 6 demonstrate that protein species with different number of reactive sulfhydryls can be separately determined by the PAGE method, while conventional optical method gives only an average number of reactive sulfhydryls per protein.

Analysis of ovalbumin synthesized in a cell-free translation system

Ovalbumin synthesized in a cell-free translation system was analyzed for its sulfhydryls and disulfides. Hen oviduct mRNA was translated in wheat germ extracts in the presence of [^{35}S]methionine. The translation products were alkylated by the one-step procedure, and electrophoresed on high-pH urea gel. By fluorography a ladder consisting of seven bands was detected as the major radioactivity (Fig. 7, lane d). These bands corresponded to newly translated ovalbumin species with different introduced number of IAA-carboxyls, since their mobilities were

consistent with those of authentic dephosphorylated ovalbumin, and since these translated proteins showed the binding ability to anti-ovalbumin antibody column (data not shown). Other translated egg white proteins detected by sodium dodecyl sulfate PAGE (data not shown) showed quite different mobilities from ovalbumin in the high-pH denaturing gel.

The possibility for disulfide bond formation in ovalbumin during translation in the cell-free system was examined. Ovalbumin was synthesized in varying concentrations of oxidized glutathione, and alkylated by the two-step procedure. No
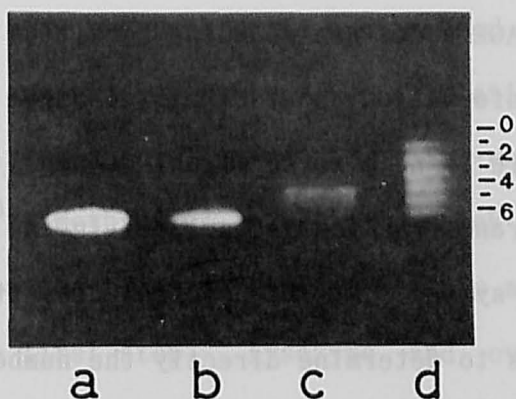

Fig. 7. Analysis of ovalbumin translated in a cell-free system.
    Hen oviduct mRNA was translated in a cell-free wheat germ system in the presence of radiolabeled methionine and varying concentrations of oxidized glutathione (lanes a and d, 0 mM; lane b, 2.0 mM; lane c, 4.0 mM) in a final volume of 20 µl.   Parts (3 µl) of the mixtures were withdrawn and acid-precipitable radioactivities were counted. Other parts (2 µl) of the translation products were alkylated by the two-step procedure in lanes a, b, and c, and by the one-step procedure with varying ratios of IAA to IAM in lane d.   Acid-precipitable radioactivities corresponded to 99,000, 84,000, 56,000, and 530,000 dpm in lanes a, b, c, and d, respectively.   Proteins were visualized by fluorography.   Exposure of X-ray film was 24 h at -70°C.

disulfide bond was formed in the absence of oxidized glutathione (Fig. 7, lane a). In the presence of 4 mM oxidized glutathione, however, ovalbumin species with four reactive sulfhydryls, hence the species with one disulfide bond, was detected (Fig. 7, lane c).

Although the importance of studying folding mechanism of elongating peptides during translation has been pointed out (36), neither excellent _in vitro_ system nor useful analytical method has been available. In previous studies (38,39,40), cell-free translation system required a microsomal membrane fraction for the signal processing of a newly synthesized protein; sodium dodecyl sulfate PAGE under non-reducing condition that does not provide direct information about disulfide bond formation was employed. There is no cleaved signal sequence in ovalbumin; no microsomal membrane fraction is included in the present cell-free translation system. The present two-step alkylation procedure allows us to determine directly the number of disulfide bonds and the reactivity of cysteine residues. We are now addressing the folding problem of ovalbumin translated in the cell-free system by monitoring the state of half-cystines as a probe for protein conformation.

The preceding data was based on the fact that in the two-step alkylation method, proteins migrated at a speed in direct proportion to the ratio of molecules of IAA introduced to those

of IAM introduced, which reflected the numbers of reactive sulfhydryl groups in the proteins tested. Proteins fully alkylated with IAA or IAM can be prepared, if the proteins are modified by the same reagent at the two alkylation steps. If the protein tested contains some cleaved disulfides, the mobility of the protein modified with different agents at the two steps should be between the two extremes. Therefore, if the proteins tested are modified with the four different combinations of the two alkylating agents in the two steps (IAA/IAA, IAA/IAM, IAM/IAA, and IAM/IAM), the sulfhydryl-to-disulfide ratio can be found by comparison of the mobilities of the four samples.

Lysozyme and ovotransferrin synthesized in chick oviduct explants were analyzed after two-step alkylation with the four different combinations of IAA and IAM. Egg-white proteins were labeled with [^{35}S]methionine in isolated chick oviduct explants, solubilized from the tissues, and immediately alkylated with IAA or IAM in the first step. Lysozyme and ovotransferrin were purified, fullyreduced with an excess of DTT, and alkylated with IAA or IAM in the second step. Proteins were analyzed by gel electrophoresis and fluorography. The mobility of radiolabeled lysozyme modified with IAA in the first step and with IAM in the second step of alkylation (Fig. 8a, lane d) was exactly the same as that of the sample modified with IAM alone in the two steps of alkylation (lane e), and the mixture of these two samples gave a

single band (lane f). When radiolabeled lysozyme was modified
with IAM and IAA in that order (lane c), its mobility was the
same as that of the sample modified with IAA alone in both steps
(lane b). A mixture of the samples used in lanes b and c gave a
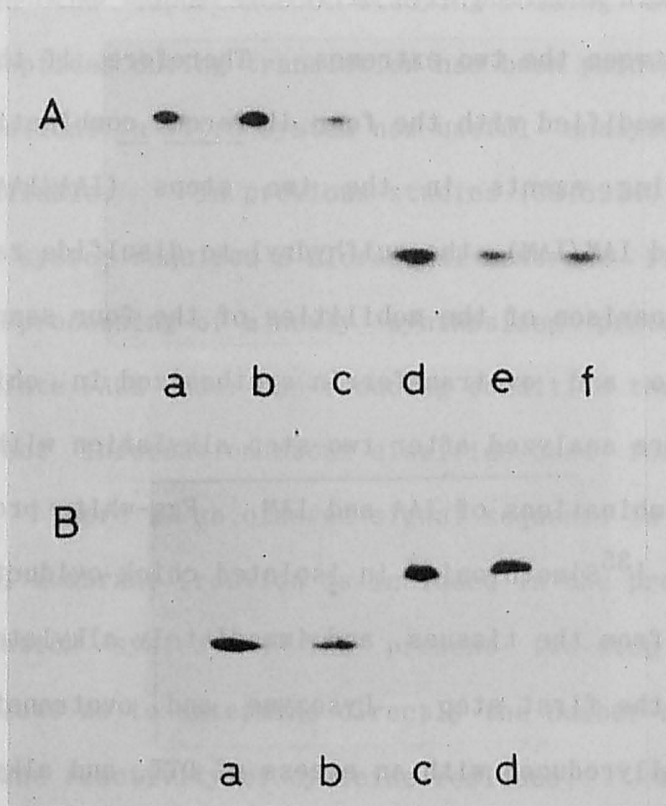single band (lane a). Therefore, the radiolabeled lysozyme,

Fig. 8. Analysis of lysozyme and ovotransferrin synthesized in
isolated chick oviduct explants. Egg-white proteins were labeled
for 15 min with [^{35}S]methionine in isolated chick oviduct explants,
solubilized from the tissues, and immediately alkylated with IAA in
the first step (A, lanes b and d; B, lanes b and d) or with IAM (A,
lanes c and e; B, lanes c and e). In (A) lysozyme was purified by
cation-exchange chromatography, fully reduced with 5 mM DTT, and
alkylated with IAA (lanes b and c) or with IAM (lanes d and e) in the
second step. Lanes a and f have a mixture of the samples of lanes d
and e,respectively. In (B) ovotransferrin was purified by use of
immobilized anti-ovotransferrin IgG, fully reduced with 5 mM DTT, and
alkylated with IAM (lanes a and b) in the second step. Following
acid-urea PAGE, proteins were made visible by fluorography.

when modified with different reagents in the two steps, migrated depending only on the reagent used in the second step. The same was true with radiolabeled ovotransferrin (Fig. 8B). These data indicated that at least under the conditions used here the formation of intramolecular disulfide bonds in both lysozyme and ovotransferrin was completed. If the proteins tested are modified as in Fig. 8 with four different combinations of IAA and IAM in the two steps, the extent of disulfide bond formation can be determined by compairing the mobilities of the four samples without any additional standard protein.

In conclusion, we believe that the present PAGE procedure provides a general method for the determination of protein sulfhydryls and disulfide in translation products as well as in isolated proteins. If a non-purified sample consists of a set of proteins that can be separated with one another on the denaturing gel, the sulfhydryls and disulfides in each protein should be determined independently. If this is not the case, use of a specific staining, such as Western blotting, is expected to work effectively.

CHAPTER 2

Conformational analysis and renaturation of ovotransferrin :
A protein containing many disulfide bonds

Refolding processes of the denatured and disulfide-reduced proteins are important not only theoretically, but also practically, since the recovery and the reactivation of recombinant proteins expressed in bacterial cells are difficult (41).   Disulfide bond formation has been investigated in detail with relatively small proteins but little is known about the refolding mechanism of a large protein that consists of a complex multidomain structure and many disulfides except for the individual domains of bovine serum albumin (42).

As a model of complex proteins which contain many disulfide bonds and consist of multidomains, ovotransferrin, which is one of the egg white proteins, was examined on its refolding process. The protein consists of a single polypeptide chain with a molecular mass of about 78,000 Da, and contains 15 intramolecular disulfide bonds (43).   This protein can be separated to two half-molecules by limited proteolysis with trypsin (18). Ovotransferrin has single carbohydorate chain on the part of C-terminal half-domain.   Therefore, N-terminal half-domain does not have carbohydrate chain but C-terminal half-domain has.   As for the disulfide bonds, N- and C- terminal half-domains have 6

and 9 bonds respectively. Thus, the half-molecules have a few points different feature each other beside a common motif on their conformation evaluated from the three dimensional structure of related protein : lactoferrin (44). Each half-molecule has similar, compact structure and has an iron-binding site independently. This iron-binding ability of each half-molecule can be regarded as a probe of the native conformation. In the refolding process of the reduced and denatured ovotransferrin, incorrectly paired disulfide formation is unavoidable and result in the formation of scrambled intermediates. Additionally, coagulation mediated by hydrophobic interactions would occur on reduction of the disulfides in ovotransferrin (45). These disadvantages are supposed in the refolding process of ovotransferrin.

In this chapter, optimal conditions required for efficient refolding of ovotransferrin are sought to avoid coagulation and the formation of scrambled intermediates. 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. Practically, it requires two steps: in the first step, reduced and low temperature conditions lead the protein to the native-like structure, and in the second step, oxidized and higher temperature conditions confirm the regeneration of the correct

disulfide bonds. The reduced and denatured form of ovotransferrin achieved efficient refolding according to this two-step scheme, which contained the formation of the native-like structure in the reduced and non-denaturing conditions.

MATERIALS AND METHODS

Materials

Ovotransferrin and its half-molecules (N-terminal and C-terminal half-molecules) were prepared as described (18). Reduced and oxidized glutathione were purchased from Kohjin. Other chemicals were guaranteed grade of 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 EDTA) at 37 $^{\circ}$C for 30 min, and then alkylated with 50 mM IAA at 37 $^{\circ}$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.

Effects of Anions on the Chemical Reduction of Disulfide Bonds

The N-terminal and C-terminal half-molecules (0.25 mg/ml) were incubated at 37°C for 30 min with 2 mM DTT in 0.1 M Tris-HCl buffer, pH 8.0 containing different concentration s of anions.

The sulfhydryl contents of proteins after incubation with DTT were measured by the photometric method of Ellman (21) as described (46).

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 ^{o}C for 30 min in Buffer A. Refolding was initiated by a 100-fold dilution of the reduced, denatured proteins with a refolding buffer. All the buffers were degassed at reduced pressure prior to the experiments. The reduced, denatured proteins were freshly prepared on every cycle of refolding experiments.

Disulfide Bond Analysis

The reduced, denatured forms (2.0 mg/ml) of whole ovotransferrin and its half-molecules were diluted 100-fold at 0 ^{o}C with Buffer B (0.1 M Tris-HCl, pH 8.2, 1.0 mM EDTA) containing 1.0 mM GSH and 0.5 mM GSSG, and incubated at 37 ^{o}C for distinct period. Disulfide bonds in the proteins were mainly analyzed by a method which includes two-step alkylation and PAGE described in chapter 1, since protein species with different numbers of disulfide bonds can be distinctly detected by this technique. In order to analyze the whole molecule of ovotransferrin with 15 disulfide bonds, the method was modified in the second alkylation

reagent.   For   the better resolution on the PAGE gel,   on   the

contrary to IAA with negative charge, 4,4'-vinylpyridine was used

and positive charge was introduced.   Briefly, the   reoxidization

was terminated by trapping free sulfhydryls with an alkylation

reagent.   The alkylation was performed by the incubation of

whole ovotransferrin with 25 mM IAA, and of the half-molecules

with the same concentration of IAA or IAM at 30 ^{O}C for 10 min.

To the mixture, 0.1 volume of the alkylated trypsin inhibitor

(4.0 mg/ml) and 10 volumes of cold acetone, 1 N HCl (98:2) were

added , and then proteins were precipitated by centrifugation

(3,000 X g, 5 min).   After the precipitates were washed three

times by repeated resuspension in cold acetone, 1 N HCl, H_2O

(98:2:10) and centrifugation, proteins were dissolved in Buffer

A, fully reduced by an incubation with 10 mM DTT, and modified

with a different alkylation reagent from the one used for the

termination of the reoxidization (ovotransferrin, 0.1 M 4,4'-

vinylpyridine at 37 ^{O}C for 60 min; half-molecules, 25 mM IAM at

37 ^{O}C for 15 min).   Low pH urea PAGE gel was carried out as

describrd in chapter 1.

Preparation of standard proteins with different numbers of
disulfide bonds

     The N-terminal half-molecule of ovotransferrin was reduced

with different molar ratios of DTT to half-molecule: 0, 1.5, 3.0,

4.5, and 70. The samples were alkylated with IAM in the first step and with IAA in the second step, and then mixd altogether. The C-terminal half-molecule was reduced in the molar ratios for DTT to protein of 0, 1, 3, 5, 7, 70. The samples were alkylated by the two-step procedure and mixed in the same way. Whole ovotransferrin was reduced in the molar ratios for DTT to protein of 0, 3.3, 6.5, 10, 13, and 40. These samples were alkylated with IAA in the first step and with 4,4'-vinylpyridine in the second step, and mixed.

Two-step procedure for renaturation

The reduced, denatured forms (2.0 mg/ml) of whole ovotransferrin and its half-molecules were diluted 100-fold at 0 ^{0}C with Buffer B containing 1 mM GSH, and preincubated at 0 ^{0}C for 5 min. Reoxidization was initiated by the addition of 0.025 volume of GSSG giving an final concentration of 0.5 mM, and the mixture was allowed to stand for different times at 22 ^{0}C. Then, the reoxidization was terminated by trapping free sulfhydryls with an alkylation reagent and treated by the two-step alkylation method as a former section except that the order of alkylation reagents was reversed in the experiment of the half-molecules.

RESULTS AND DISCUSSION

Conformation of N-terminal and C-terminal half-molecules

The two half-molecules, which are separated by limited proteolysis with trypsin, has similar but slight different structure. Comparison between the two half-molecules can provide any information on their conformation. Typically, only C-terminal half-molecule has a sugar chain and has three additional disulfide bonds. However, iron binding capacity is the common feature of them and their disulfide topology is similar to each other besides the three additional disulfide bonds on the C-terminal half. Iron-binding capacity requires co-presense of anion for each half-molecules and anion-binding has been confirmed practically for serum transferrin (47). Iron-binding in the presence of anion has demonstrated to make both the half molecules be markedly resistant against protease attack (48) and disulfide reduction by a thiol (49). The extent of disulfide reduction of each domain with DTT was investigated in the presense of anion without iron. The number of free sulfhydryl groups after treatment with DTT with different concentrations of phosphate was quantitated by Ellman method (fig. 9). The disulfide reduction of both the half-molecules depended on the concentration of phosphate. More predominant protection by phosphate was observed for the N-terminal half-
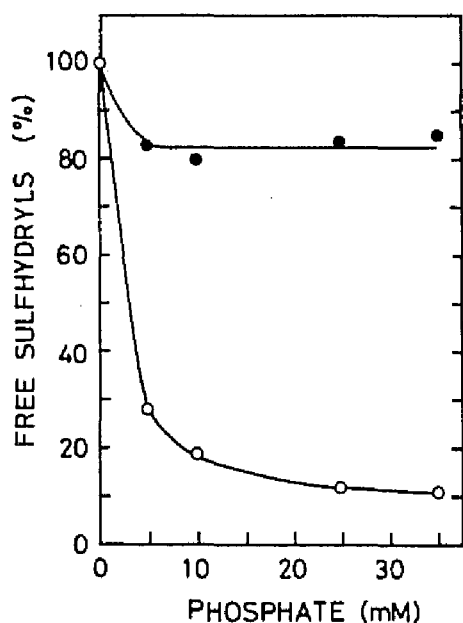
Fig. 9.   Effect of concentration of phosphate on the reduction of disulfide bonds in the two half-molecules.   The N-terminal half-molecule (O) and the C-terminal half-molecule (●) were reduced in the presence of various concentrations of phosphate with 2 mM DTT for 30 min at 37°C and then analysed as to their free sulfhydryl contents by the method of Ellman.   The free sulfhydryl contents of the N-terminal and C-terminal half-molecules in the absence of phosphate were 9.1 and 11.4 mol per/mol of the protein, respectively.   The ordinate represents percentages as to these values.

molecule than for the C-terminal one.   Although anions do bind

to the C-terminal half-molecule, the anion-dependent protection

was very little for this half-molecule.   X-ray crystallographic

studies have shown that an arginine residue may play an important

role as the anion-binding site in the iron-binding cleft (44,50).

Recently, another positively charged residue, lysine, has been

pointed out to be situated within 6 A of the iron position in

each of the two domains of serum transferrin (50).   This lysine

is replaced by glutamine in the C-terminal half-molecule of ovotransferrin. Such a difference in the positively charged residue in the iron-binding cleft might be related to the differential anion effects on the overall conformations of the two half-molecules. The protection effect against protease and thiol could be expressed by stabilization of the conformation of each half-molecule. The following experiments for renaturation of the whole and the two half-molecules of ovotransferrin were performed in the absence of iron and such an anion.

Renaturation of ovotransferrin by conventional single-step reoxidization

The reduced and denatured ovotransferrin and its half-molecules were attempted to refold into the native conformation. This process was evaluated according to the time course of the disulfide bond formation by the method described in chapter 1. with a slight modification. Since the conbination of IAA and IAM in the two-step alkylation had not get sufficient resolution for the whole molecule of ovotransferrin, neutral IAM was replaced by 4,4'-vinylpyridine, which would introduce positive charge into the protein in contrast to IAA-negative charge. This made the difference in the net charge of the protein tested twice compared to the combination of IAA and IAM. Consequently, the difference in the mobility on the page gel was increased.

Figure 10 (W) shows good resolution of 16 bands from 15 disulfides of the whole molecule of ovotransferrin was achieved by the combination of IAA and 4,4'-vinylpyridine.

Firstly, the whole molecule of ovotransferrin and its half-molecules were attempted to regain their native conformation by a normal procedure, which has been used for proteins with low molecular weight or without disulfide bonds. The whole molecule and the half-molecules were reduced by incubation with DTT in the denaturing conditions containing 8 M urea. Then, the mixtures were diluted 100-fold at 30^{0}C with refolding buffer containing 1 mM GSH and 0.5 mM GSSG. After distinct time period, aliquots of the diluted mixture were drawn and terminated the reoxidization by blocking free sulfhydryl groups with IAA. The disulfide bonded sulfhydryl groups were blocked by 4,4'-vinylpyridine for the whole molecule and by IAM for the two half-molecules after reduction of disulfide bonds with DTT in urea-containing buffer. Figure 1. shows the whole molecule (W) and the half-molecules of ovotransferrin (N and C) did not complete their disulfide bonds formation even in 20 hr. After 20 hr-reoxidization, the N-terminal and C-terminal half-molecules of ovotransferrion contained at most 5 and 8 disulfide bonds, respectively, as well as whole molecule contained 13 or 14 disulfide bonds. In this usual renaturation procedure, hydrophobic interaction among unfolded molecules and incorrect disulfide bonds may occur and
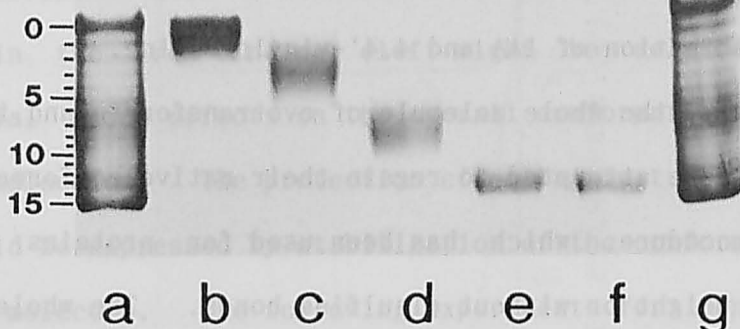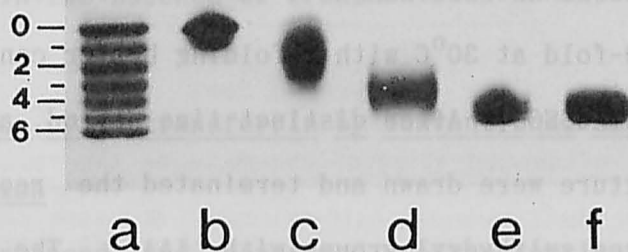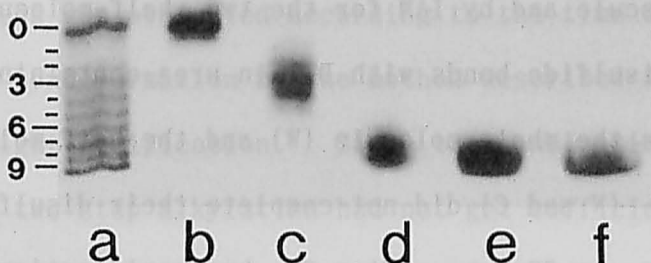
Fig. 10. Time courses of the formation of disulfide bonds of whole ovotransferrin and its half-molecules with no preincubation. The reduced, denatured forms of the N-terminal half-molecule (N), the C-terminal half-molecule (C), and whole ovotransferrin (W) were diluted and reoxidized at 22 °C in Buffer B containing 1.0 mM GSH and 0.5 mM GSSG for different times (min); lane b, 0; lane c, 5; lane d, 30; lane e, 180; lane f, 1200. The numbers of disulfide bonds were analyzed by the two-step alkylation and PAGE techniques. The alkylation of the two half-molecules were 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. The alkylated samples were electrophoresed on the low-pH urea gel as described in chapter 1.

-40-

cause irreversible coagulation.

   In order to avoid coagulation, although, hydrophobic interaction and incorrect disulfide bond formation must be suppressed inter-molecularly. At low temperature and low protein concentration, hydrpophobic interaction could be repressed between molecules. Protein folding with dilution of denaturant must be proceeded in low temperature and low protein concentration. Correct disulfide bond formation could be achieved by disulfide bond formation after spontaneous folding, because a pair of sulfhydryl groups which is originally disulfide-bonded in the native molecule might be placed neighboring to each other in the folding process. Additionally, the folding must be proceeded in reducing conditions to avoid incorrect disulfide bond. After spontaneous folding under the reducing conditions at low temperature, original disulfide bonds could be regenerated effectively. Consequently, the optimal conditions for the refolding of the fully reduced and denatured ovotransferrin and its half-molecules were determined as a following two-step procedure. At first step, the reduced and denatured protein in the concentrated denaturant is diluted with buffer solution containing 1.0 mM GSH and preincubated at 0 ^{o}C for 5 min; according to this scheme the protein is supposed to recover its native-like conformation but original disulfide bonds. At subsequent second step, GSSG is added to the reaction

mixture giving final concentration of 0.5 mM; pairs of the half-cystine residues which had been bonded in the native protein also regain the original disulfide bonds correctly.

The deduced native-like structure after the preincubation at low temperature has been examined by far-UV CD measurement (51). In adition to the protein which recovered its native-like structure by the preincubation, a protein species fully reduced in non-denaturing conditions has been prepared and analyzed. CD spectrum of this reduced ovotransferrin exhibits a slight difference from that of the native protein but significant value of molecular ellipticity reveals rather extent of secondary structure has existed in this molecule. From this feature, a partially folded structure has been suggested and related to the "molten-globule" like structure. Additionally, in this deduced partially folded structure, sulfhydryl groups may be prevented from incorrect pairing and each original pair of sulfhydryls may be drawn closely.

The refolding processes of the whole ovotransferrin and its half-molecules according to this two-step procedure were confirmed by monitoring the time courses of the second step reoxidization after first step preincubation. According to the distinct reoxidization period, aliquot of refolding sample was drawn and reoxidization was terminated by blocking of free sulfhydryls with alkylating reagents. As shown in figure 2.,
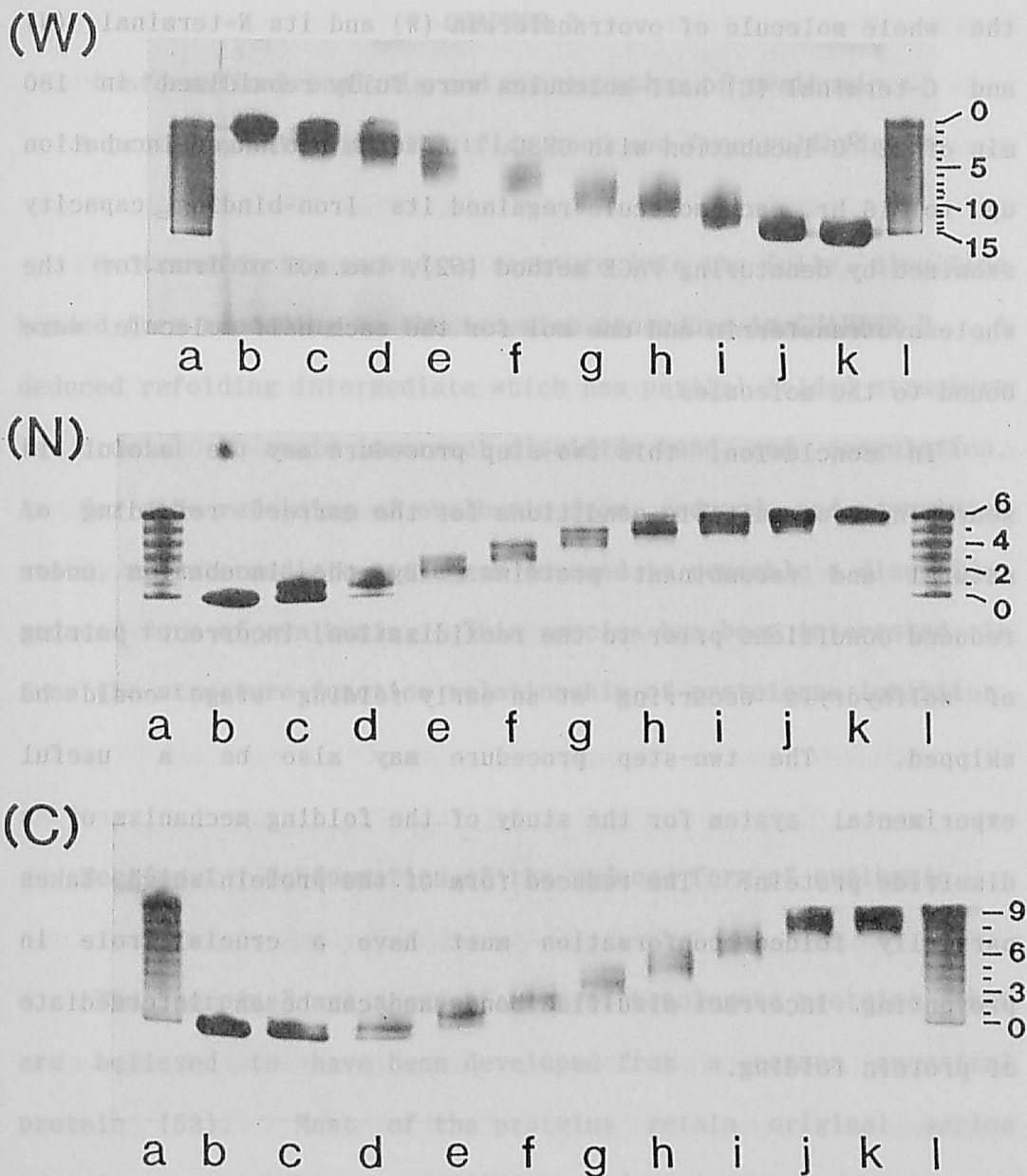
Fig. 11   Time courses of the formation of disulfide bonds of whole ovotransferrin and its half-molecules after preincubation in low-temperature and reduced conditions.   The reduced, denatured forms of the N-terminal half-molecule (N), the C-terminal half-molecule (C), and whole ovotransferrin (W) were 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 b, 0; lane c, 1; lane d, 2; lane e, 4; lane f, 6; lane g, 9; lane h, 13; lane i, 20; lane j, 60; lane k, 180.   The numbers of disulfide bonds were analyzed by the same procedure in Fig. 10.

the whole molecule of ovotransferrin (W) and its N-terminal (N) and C-terminal (C) half-molecules were fully reoxidized in 180 min of 22 ^{O}C-incubation with GSSG. After a prolonged incubation up to 16 hr, each molecule regained its iron-binding capacity examined by denaturing PAGE method (52), two mol of iron for the whole ovotransferrin and one mol for the each half-molecule were bound to the molecules.

In conclusion, this two-step procedure may be useful in searching for suitable conditions for the correct refolding of natural and recombinant proteins. By the incubation under reduced conditions prior to the 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. The reduced form of the protein which takes partially folded conformation must have a crucial role in preventing incorrect disulfide bonds and can be an intermediate of protein folding.

CHAPTER 3

Conformatioal analysis and renaturation of ovalbumin :

A protein containing disulfide bond and free sulfhydryls

Ovotransferrin proved to renature into the fully disulfide bonded form according to the two-step procedure in CHAPTER 2. A deduced refolding intermediate which has partial folded structure is crucial to avoid incorrect disulfide bond and coagulation. As for the refolding of ovalbumin from reduced and denatured state, an intermediate state was assumed to resemble a disulfide-reduced form of ovalbumin. This species has been interested in from the structure-function relationship of proteinase inhibitor.

Section 1   Conformation of the reduced form of ovalbumin

The "serpins" are a superfamily of homologous proteins that are believed to have been developed from a common ancestral protein (53). Most of the proteins retain original serine proteinase inhibitor activity (α_1-antitrypsin, α_1-antichymotrypsin, antithrombin III etc.), but the rest have lost the function as the proteinase inhibitor (ovalbumin, angiotensinogen and thyroxine binding protein). Although the homology among the superfamily is about 30%, all members have

been hypothetically deduced to have similar conformation to α_1-antitrypsin, a typical serine proteinase inhibitor, since the protein in its nicked form has been the only example, in which the molecular structure has been constructed based on the X-ray crystallographic analysis (54). Very recently, however, three dimensional structure of ovalbumin has been resolved both in the native (55) and the nicked form (56), thereby structural similarity to α_1-antitrypsin has been confirmed. Since ovalbumin has no inhibitor activity, the egg white protein along with α_1-antitrypsin is a useful model protein for the study of the relationship between structure and activity of the serine proteinase inhibitors.

Ovalbumin has an intramolecular disulfide bond and this is not a common feature to the serpins; α_1-antitrypsin contains no intramolecular disulfide (53). The role of the disulfide bond has not been established in relation to the stability and conformation of ovalbumin. Generally, small disulfide proteins consisting of a single domain take fully unfolded or native-like conformation in their disulfide-reduced forms. Bovine pancreatic trypsin inhibitor and RNase A unfold upon the reduction of their intramolecular disulfides (57,58) ; in contrast, the disulfide-reduced forms of Fc fragment of immunoglobulin and human growth hormone take conformation indistinguishable from the disulfide-bonded forms (59,60). With regard to a multidomain protein, the

fully reduced form of ovotransferrin takes an intermediate conformation between the native and unfolded conformation (51).

In this section, the conformation of the reduced ovalbumin was examined. The overall conformation of the reduced form of ovalbumin was evaluated by CD spectrum, resistance to protease digestion and the reactivity of cysteine sulfhydryls against alkylation reagents. Here we report that the disulfide-reduced form of ovalbumin takes native-like conformation, except that local conformation around the two sulfhydryl groups that are originally involved in the disulfide bond is slightly loosened in the reduced form.

MATERIALS AND METHODS

Materials

Ovalbumin was purified from fresh egg white by crystallization in ammonium sulfate solution (61) and recrystallized three times. Dephosphorylated ovalbumin was prepared by acid phosphatase treatment and ion-exchange chromatography as described elsewhere (19). Trypsin and subtilisin-BPN' were purchased from Sigma. The reduced form of ovalbumin was prepared under non-denaturing conditions by incubating the native protein at 1.0 mg/ml with 15 mM dithiothreitol (DTT) at 37^{0}C for 2 h in Buffer A (50 mM

tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.2 /1 mM
Na$_2$ethylenediamine tetraacetate (EDTA)).

Analysis of the number of the reactive cysteine sulfhydryls by polyacrylamide gel electrophoresis

The numbers of disulfide bonds and reactive sulfhydryl groups in dephosphorylated ovalbumin were determined by selective two step alkylation and following polyacrylamide gel electrophoresis (PAGE) method described in CHAPTER 1.

Identification of reactive sulfhydryl groups

The location of reactive sulfhydryls in the reduced form of ovalbumin was determined by a sequence analysis after protein alkylation with IAEDANS and fragmentation with trypsin followed by gas-phase sequencing. The reduced form of dephosphorylated ovalbumin (0.5 mg) was alkylated with 15 mM IAEDANS at 37°C for 10 min in 1 ml of Buffer A containing 5 mM DTT. Excess IAEDANS were trapped by incubating with 18 mM DTT at 37°C for 5 min. The alkylated ovalbumin was mixed with 10 volume of cold acetone/ N-HCl(98/2), kept at -20°C for 1 h, precipitated by centrifugation (3,000 x g, 10 min), and then washed three times with cold acetone/ N-HCl/ H$_2$O (98/2/10). The alkylated protein was dissolved in 120 μ l of 0.1 M Tris-HCl buffer (pH 8.2) containing 6 M urea and incubated at 50°C for 10 min. After urea

concentration was diluted to 2 M by the addition of 240 μ l of 0.1M Tris-HCl buffer (pH 8.2), digestion was immediately performed at 30°C for 3 h with trypsin in an ovalbumin to the protease ratio of 100/1 (w/w). The mixture (120 μ l) was applied to a reverse phase HPLC column (YMC AP-302), and peptides were eluted with an acetonitrile linear gradient (0-80%) in 0.1% trifluoroacetic acid (pH 2.0). Peptide peaks exhibiting IAEDANS fluorescence (Excitation 340 nm ; Emission 510 nm) were collected and further purified by the same reverse phase HPLC with an acetonitrile linear gradient (20-80%) in 0.5M triethylamine /acetic acid buffer (pH 5.0). The purified peptides were analyzed for amino acid compositions with an amino acid analyzer (Hitachi, model 835-30) and for primary sequences with an gas-phase protein sequencer (Applied Biosystems, model 477A/120A).

CD spectrum measurement

CD spectra were recorded with a J-500C spectropolarimeter (JASCO). The far-UV CD spectra of the native and the reduced ovalbumin were measured at 1 mg/ml in 20 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM Na$_2$EDTA with a 0.1-mm cell in a wavelength range from 190 nm to 250 nm. DTT in the reduced ovalbumin solution was removed with a gel filtration column (PD-10, Pharmasia-LKB-Biotechnology) prior to CD measurements. The near-UV CD spectra (250 nm to 340 nm) of the native and the

reduced ovalbumin were measured at 1.0 mg/ml in 50 mM Buffer A with a 10 mm cell. The data of the far-UV CD spectra were expressed in mean residue ellipticity (degree cm^2/decimol), and those of the near-UV CD spectra, in molar ellipticity.

Susceptibility to protease digestion

Since the native, disulfide-bonded form of ovalbumin is highly resistant to trypsin, we used the susceptibility to the protease as a probe for evaluation of the conformation of the disulfide reduced form. Ovalbumin, in the disulfide-bonded or in the disulfide-reduced form, was incubated at 0.2 mg/ml with various concentrations (0-0.5 mg/ml) of trypsin at 25^oC for 1 min. The digestion was stopped by the incubation at 0^oC for 5 min with soy bean trypsin inhibitor, the concentration of which was twice of that of the protease. The proteins in sodium dodecyl sulfate (SDS)-PAGE sample buffer (62.5 mM Tris-HCl pH 7.0/ 1% SDS/ 10% glycerol/ 20 mM 2-mercaptoethanol) were pretreated in boiling water bath for 2 min, electrophoresed on a SDS polyacrylamide gel (10% polyacrylamide monomer/ 0.27% N,N'-methylenebisacrylamide) according to the standard method by Laemmli (62), and then stained with Coomassie brilliant blue R250. The amount of trypsin-resistant ovalbumin was determined from the band intensity that was measured with a densitometer (Shimadzu CS-910).

The susceptibility of the reduced ovalbumin was also examined by using subtilisin. Ovalbumin solution (1.0 mg/ml) was incubated in Buffer A with 0.125 μ g/ml of subtilisin (subtilisin-BPN' type V) at 25°C in a total volume of 20 μ l. After various time periods, proteolysis was stopped by the addition of equal volume of 1.0 % trifluoroacetic acid solution. The mixture was dried in vacuo, dissolved in 100 μ l of the SDS-PAGE sample buffer, and pretreated in boiling water bath for 2 min. A part (0.1 volume) of the mixture was analyzed by SDS-PAGE in the same way, except that 15% polyacrylamide / 0.4% N,N'-methylenebisacrylamide gel was used.

N-terminal analysis of proteolytic fragments

The disulfide-reduced ovalbumin (1.0 mg/ml) was incubated in Buffer A with 0.5 μ g/ml of subtilisin at 25 ^{O}C for 3 h in a total volume of 250 μ l, then mixed with the equal volume of 1% trifluoroacetic acid solution. The sample was dried in vacuo, dissolved in 250 μ l of the SDS-PAGE sample buffer, pretreated in a boiling water bath, and electrophoresed on a 10 % polyacrylamide gel (13.5 X 13.5 X 0.1 cm).

The protein was transferred to a polyvinyliden difluoride membrane (MILLIPORE, Immobilon) with a semi-dry transfer unit (Saltoblot II S) as described (63). The proteins blotted onto the membrane were stained with 0.1 % Ponceau solution. After

washed with 1 % acetic acid solution and distilled water, the membrane was dried. Protein bands were cut and subjected to a sequence analysis with the protein sequencer.

RESULTS

Effects of disulfide reduction on the reactivity of sulfhydryl groups

Native ovalbumin was incubated with DTT under non-denaturing conditions, and the extent of disulfide reduction was evaluated by a method including two-step alkylation and PAGE described in CHAPTER 1, which enables the estimation of protein species with different number of free sulfhydryls distinctly. As shown in Fig. 12 (lane f), when ovalbumin had been pretreated with 15 mM DTT at $37^{O}C$ for 2 hr, all the six half-cystines were detected as reactive sulfhydryl groups in the presence of 8 M urea at the first-step of alkylation. The disulfide-intact ovalbumin showed four reactive sulfhydryl groups (Fig. 12, lane c). These numbers demonstrate that the disulfide bond in ovalbumin is completely cleaved under the non-denaturing pretreating conditions. Under non-denaturing conditions the four cysteine sulfhydryls in disulfide-bonded ovalbumin are all non-reactive to alkylation reagents, such as IAA. The reactivity, under non-
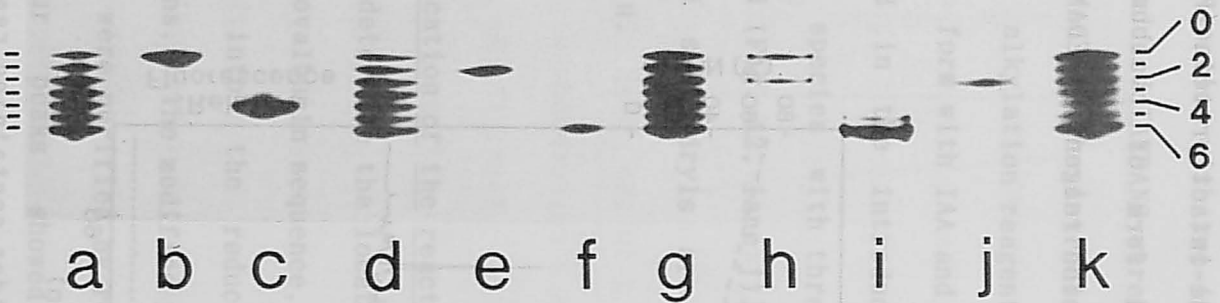
Fig. 12. Analysis of The number of reactive SH groups by PAGE. The disulfide-intact ovalbumin (lanes b and c) and the disulfide-reduced protein (lanes e,f,h,i and j) were alkylated by two-step procedure and analyzed by high-pH urea PAGE. As the standard for the correlation between protein mobilities and introduced number of negative charge, ovalbumin that had been fully reduced with 5 mM DTT in 8 M urea was alkylated with different molar ratios of IAA to IAM, and then samples were mixed (lanes a,d,g and k). The numbers on the right side indicate the introduced number of negative charges. First step alkylation with IAA was achieved in the presence (lanes c and f) and in the absence (lanes b and e) of 8 M urea. For monitoring the IAEDANS introduction, the reduced ovalbumin was alkylated with IAEDANS at the first step in the presence (lane i) and absence (lane h) of 8 M urea. In lane j, at the first step alkylation, the reduced form of ovalbumin was alkylated with 15 mM IAA and 15 mM IAEDANS in the absence of urea. Electrophoretic conditions were the same as described before (13).

denaturing conditions, of the six sulfhydryls of the reduced ovalbumin was analyzed by the same PAGE method. When ovalbumin was incubated at the first alkylation step with IAA, only one of the six sulfhydryl groups of the disulfide-reduced protein was modified with the anionic alkylation reagent, but no reactive

sulfhydryl was detected in the disulfide-intact protein (Fig. 12, lanes e and b). When the reduced form of ovalbumin was incubated with another anionic alkylation reagent, IAEDANS, the
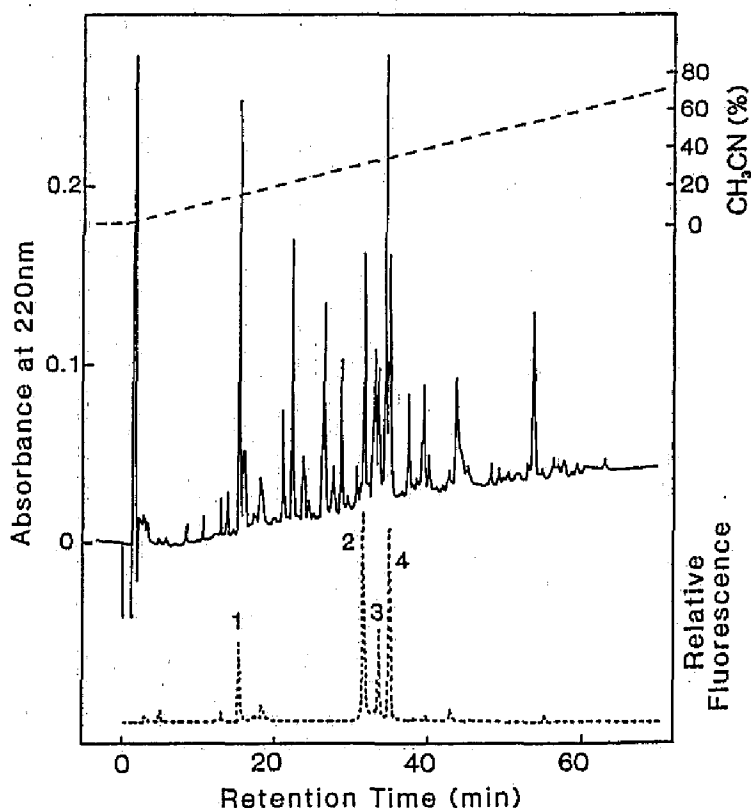

Fig. 13. Isolation of tryptic peptides containing reactive sulfhydryl groups. The reactive cysteine sulfhydryls in the reduced ovalbumin were modified with 15 mM IAEDANS, then the protein was digested with trypsin in the presence of 2 M urea. The digest was applied to a column (YMC AP302 ODS, particle size 5 μm, pore size 300Å, 4.2 x 150 mm) equilibrated with 0.1 % TFA. Peptides were eluted with a linear gradient of acetonitrile (0 to 80%)(slashed line). The flow rate was 1.0 ml/min. Peptides were detected by measurements of an absorption at 220 nm (solid line) and fluorescence emission at 520 nm (dotted line).

protein species with two introduced IAEDANS as well as those with no introduced IAEDANS were detected (Fig. 12, lane h).　Although the reason why the introduced number is different between the two anionic alkylation reagents is not clear, the incubation of the reduced form with IAA and IAEDANS at the first-step alkylation resulted in the introduction of two alkylation reagent; no protein species with three introduced alkylation reagent was detected (Fig. 12, lane j).　Thus, it can be concluded that two cysteine sulfhydryls are reactive in the reduced form of ovalbumin.

Identification of the reactive sulfhydryls

　　To determine the location of the reactive sulfhydryl groups in the ovalbumin sequence, we introduced the fluorescent probe, IAEDANS into the reduced ovalbumin under non-denaturing conditions.　The modified protein was digested with trypsin, and peptides were purified by reverse phase HPLC.　As shown in Fig. 13, four peaks showed the fluorescence of IAEDANS. Each of the four fluorescent peaks was further purified by rechromatography using the same reverse phase HPLC.　The four peaks were first characterized by amino acid analysis.　Peak 1 was found to be a non-peptide substance, since no amino acid was detected in the peak.　The other three peaks were determined for

their complete sequence from their N-terminals to C-terminals as follows:

Peak 2; FDKLPGFGDSIEAQXGTSVNVHSSLR

Peak 3;   YPILPEYLQXVK

Peak 4; LYAEERYPILPEYLQXVK

In the light of the established primary structure (64), peak 2 corresponds to the fragment, $Phe^{59}-Arg^{84}$. In this fragment Cys^{73} is included. Therefore, the X in the peak 2 can be identified as Cys^{73}. Peak 3 and Peak 4 correspond to Tyr^{111}-Lys^{122} and $Leu^{105}-Lys^{122}$, respectively. Thus, the Xs in the peak 3 and peak 4 should be Cys^{120}. Cys^{73} and Cys^{120} are both involved in the disulfide bond in the intact form of ovalbumin (65). We, therefore, concluded that the two sulfhydryl groups generated by disulfide bond cleavage, but not the other four sulfhydryls, are reactive to IAEDANS under non-denaturing conditions.

CD spectrum

It was investigated by the CD spectrum whether or not the overall conformation of the reduced ovalbumin is different from that of the disulfide-bonded one. As shown in Fig. 14, no significant difference in the far-UV CD spectrum was observed
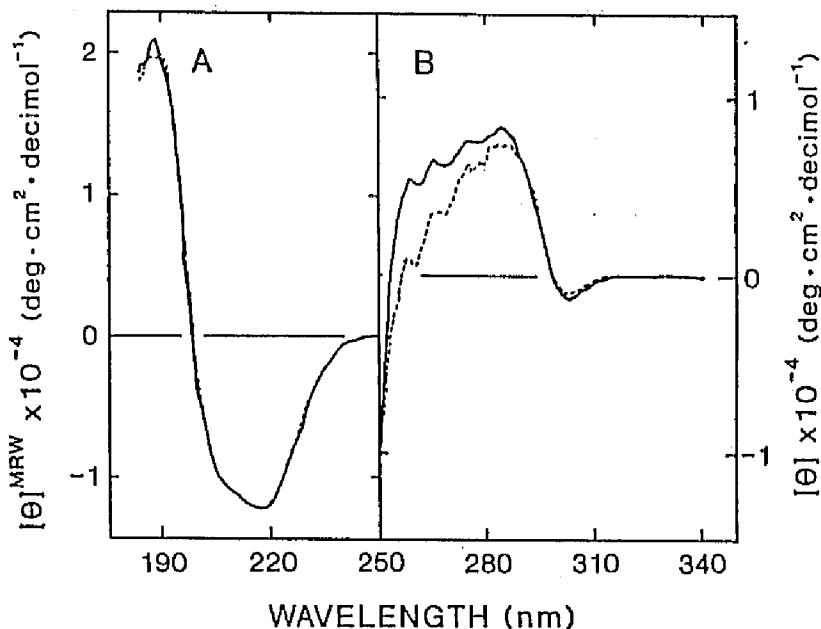
Fig. 14. CD spectra of the disulfide-intact and the reduced ovalbumin. The CD spectra of the disulfide-intact (solid line) and disulfide-reduced ovalbumin (dotted line) were measured in a far-UV region (185 nm–250 nm; panel A) and in a near-UV region (250 nm–340 nm; panel B).

between the native and the reduced ovalbumin. In contrast, in a near-UV region, the CD spectrum of the disulfide-reduced ovalbumin showed slightly reduced ellipticity at 260, 265, and 275 nm, but the wavelengths of the extrema remained unchanged in comparison to the disulfide-bonded form (Fig. 14 B). Both the positive peak at 290 nm and the negative peak at 305 nm were almost exactly the same between the two forms of ovalbumin.

Resistance to trypsin digestion

Although ovalbumin contains twenty lysine and fifteen arginine residues, it is highly resistant to trypsin in its native disulfide-bonded state (66). Heat-denatured ovalbumin, however, are quite sensitive to trypsin digestion (67).
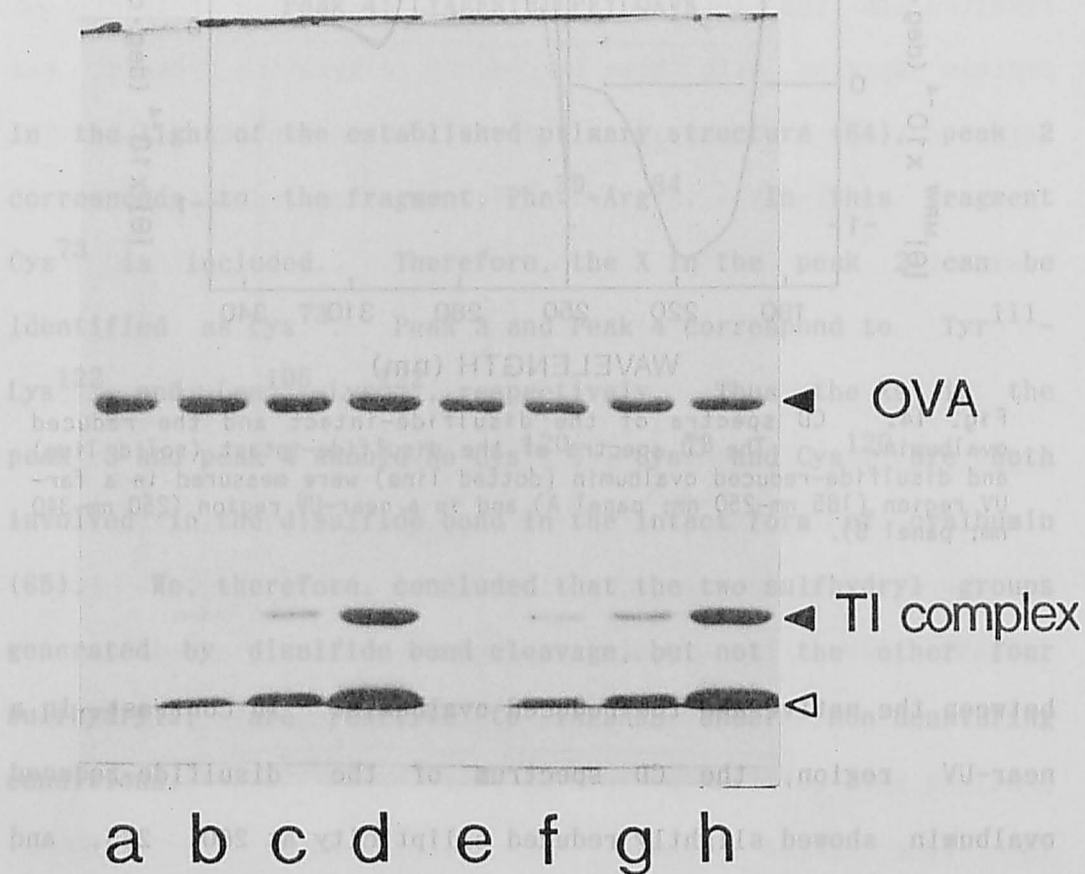

Fig. 15.   Trypsin digestion of ovalbumin.   The native (lanes a,b,c and d) and the reduced ovalbumin (lanes e,f,g and h) were incubated at 0.2 mg/ml in Buffer A (pH 8.2) with trypsin at 25 °C for 1 min.   The ratios (w/w) of trypsin to ovalbumin were 0 for lanes a and e, 0.25 for lanes b and f, 0.5 for lanes c and g, and 2.5 for lanes d and h.   The reaction was stopped by an incubation with soy bean trypsin inhibitor.   Samples were resolved by SDS-PAGE and stained with Coomassie brilliant blue R-250.   The open arrow shows the top of migration; the closed arrows of OVA and TI complex represent intact ovalbumin and the complex of trypsin and trypsin inhibitor, respectively.

Thus, trypsin resistance can be regarded as a probe of the compactness of an ovalbumin molecule. To evaluate overall protein conformation, we examined the resistance to trypsin digestion of the disulfide-reduced form of ovalbumin. As shown in Fig. 15 (lanes b-d), the disulfide-intact ovalbumin appeared completely resistant to the proteolysis by 2.5-fold (w/w) of trypsin. Surprisingly, the reduced ovalbumin was also resistant to the same high concentration of trypsin (Fig. 15, lanes f-h). These were confirmed by densitometric analysis: both of the native and the reduced ovalbumin completely retained their original amount during the trypsin digestion. Thus, in terms of trypsin resistance, the conformation of the reduced ovalbumin was indistinguishable from that of the disulfide-bonded ovalbumin.

Susceptibility to the proteolysis by subtilisin

The disulfide-bonded ovalbumin is hydrolyzed by a serine proteinase, subtilisin, at specific sites to generate a nicked form, which is called plakalbumin (66). The cleavage sites are the peptide bonds on the C-terminal side of Glu^{346}, Asp^{350} and Ala^{352}, accordingly, plakalbumin is a nicked form consisting of $AcGly^1$-Glu^{346} (42.0 k-dalton) and Ser^{353}-Pro^{385} (3.7 k-dalton), lacking a hexapeptide (Ala^{347}-Ala^{352}).

The time course of the proteolysis of ovalbumin was monitored by SDS-PAGE. Under the present conditions, we confirmed that the

disulfide-bonded ovalbumin was hydrolyzed into the two large fragments of 42.0 k- and 3.7 k- dalton (Fig. 16, panel B). These two fragments were stable until a prolonged incubation of 3 h. Further, on a sequence analysis of the sample that had been

A

(kDa)										(kDa)

55.4 —

36.5 —

20.1 —

12.5 —

6.5 —
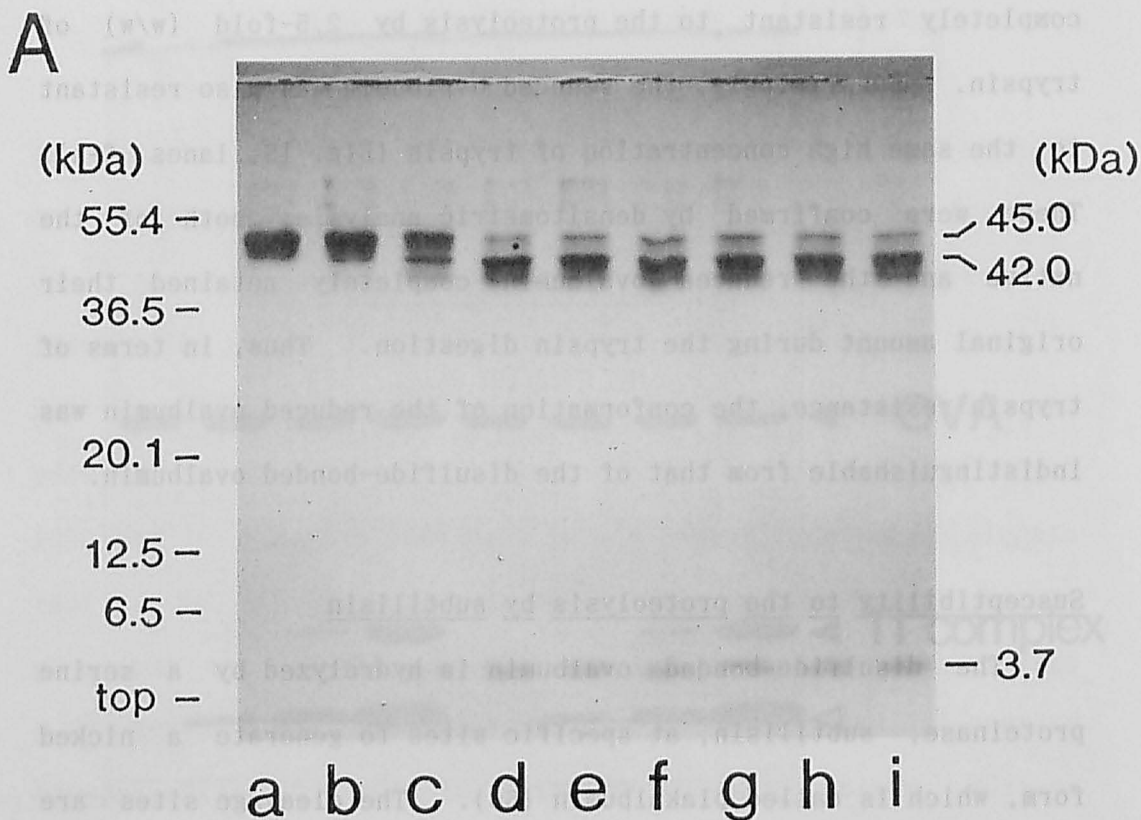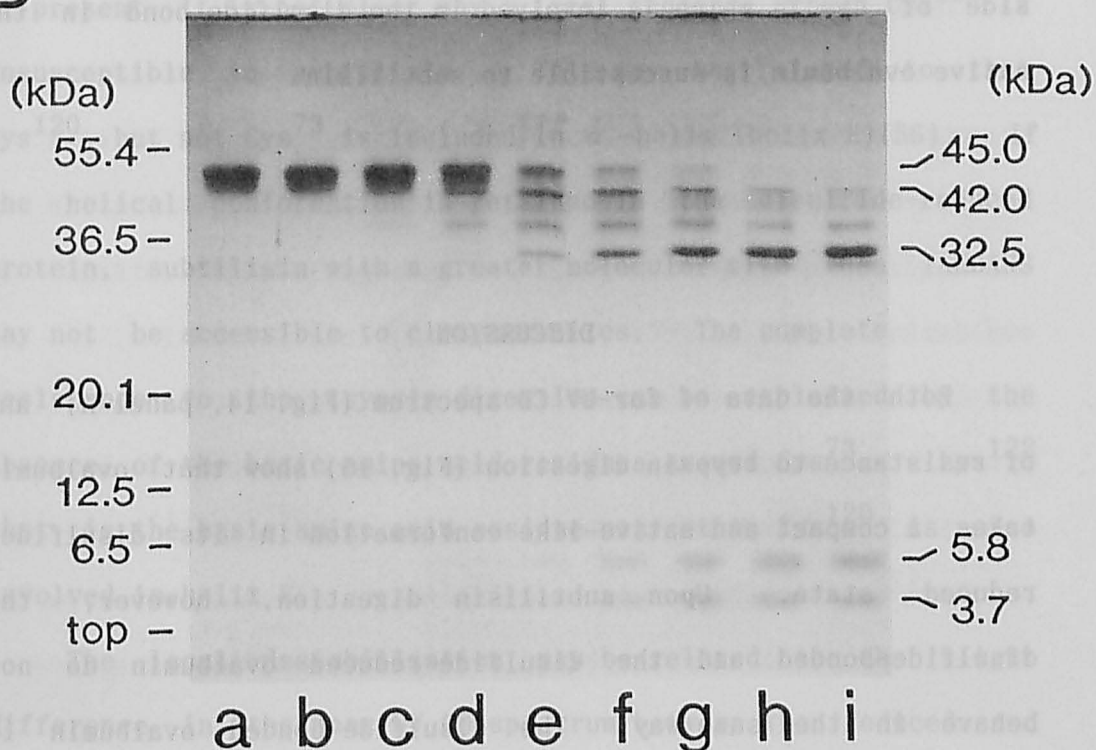
top —

45.0
42.0

— 3.7

a b c d e f g h i

Fig. 16.   Susceptibility of ovalbumin to subtilisin.   The disulfide-intact (panel A) and the disulfide-reduced ovalbumin (panel B) was incubated at 1.0 mg/ml with 0.125 μg/ml of subtilisin-BPN' for 0 (lane a), 1 (lane b), 5 (lane c), 30 (lane d), 60 (lane e), 90 (lane f), 120 (lane g), 150 (lane h), 180 min (lane i).   The reaction was terminated by the addition of an equal volume of 1% TFA.   Proteins were electrophoresed on a 15 % polyacrylamide gel and stained with Coomassie brilliant blue.   The molecular mass of the fragments were estimated from their migration using molecular weight markers (glutamate dehydrogenase, 55.4 k-dalton; lactate dehydrogenase, 36.5 k-dalton; soybean trypsin inhibitor, 20.1 k-dalton; cytochrome c, 12.5 k-dalton; bovine pancreatic trypsin inhibitor, 6.5 k-dalton).

digested for 3 h and then passed through a Sephadex G-25 column to remove a low molecular weight fragment, only a N-terminal sequence with Ser-Val-Ser-Glu-Glu-Phe-Arg-, which corresponds to the N-terminal sequence of the 3.7 k-dalton fragment was detected, suggesting that the N-terminal amino acid of the 42.0 k-dalton fragment is the acetylated glycine.

As to the disulfide-reduced ovalbumin, subtilisin digestion proceeded in such a way that temporal appearance of 42.0 k-dalton fragment was followed by the accumulation of 32.5 k-dalton fragment (Fig. 16, panel B). In addition to the same 3.7 k-

dalton fragment, a fragment with 5.8 k-dalton was also generated. The data were consistent with the idea that the 42.0 k-dalton fragment is further digested into 32.5 k- and 5.8 k-dalton fragments in the reduced form of ovalbumin. To address this possibility, we analyzed the N-terminal sequence of the newly generated 32.5 k-dalton fragment, after the peptide was transferred from SDS-PAGE gel onto a polyvinyliden difluoride membrane. The N-terminal sequence of this fragment was determined as X-Gly-Thr-Ser-Val-, which can be found in the known sequence at the location of Cys^{73} to Val^{77}. Thus, we conclude that in addition to the known cleavage sites in the disulfide-bonded ovalbumin, in the disulfide-reduced form, the N-terminal side of Cys^{73}, which is involved in the disulfide bond in the native ovalbumin is susceptible to subtilisin.

DISCUSSION

Both the data of far-UV CD spectrum (Fig. 14, panel A) and of resistance to trypsin digestion (Fig. 15) show that ovalbumin takes a compact and native-like conformation in its disulfide-reduced state. Upon subtilisin digestion, however, the disulfide-bonded and the disulfide-reduced ovalbumin do not behave in the same way. The disulfide-bonded ovalbumin is

partially cleaved by subtilisin making a nicked form, known as "plakalbumin"; there is at least one additional cleavage site in the reduced protein, which corresponds to the N-terminal side of Cys^{73}. This cysteine sulfhydryl is originally disulfide-bonded with Cys^{120} in native ovalbumin. The acquired susceptibility to subtilisin suggest a local destabilization of the conformation in the reduced protein. Essentially the same conclusion can be drawn from the data of reactivity of the sulfhydryl groups against IAEDANS: only the two cystine sulfhydryls, Cys^{73} and Cys^{120}, which are disulfide-bonded in the native protein, but not the other four free sulfhydryl groups that occur in the disulfide-bonded molecule, react with the alkylation reagent. At present, it is not clear why a local sequence around Cys^{120} is insusceptible to subtilisin. This may be related to that Cys^{120}, but not Cys^{73} is included in α-helix (helix E)(56). If the helical conformation is retained in the disulfide-reduced protein, subtilisin with a greater molecular size than IAEDANS may not be accessible to cleavage sites. The complete resistance to the trypsin digestion can be explained by the absence of the basic amino acid residues around Cys^{73}. Lys^{122} that is the basic amino acid residue nearest to Cys^{120} is also involved in helix E.

The local destabilization may be related to the slight difference in the near-UV CD spectrum between the reduced and

native proteins (Fig. 14, panel B). The positive peaks at 260, 265, and 275 nm are slightly decreased, but both the positive shoulder peak at 290 nm and the negative peak at 305 nm are retained in the reduced form. Previously, the latter two peaks have been assigned to the three tryptophan residues in ovalbumin, Trp^{148}, Trp^{184}, and Trp^{267} (68,69). Three tryptophan residues are all separated from the Cys^{73} and Cys^{120} in the three dimensional structure (56). Trp^{148} is included in helix F, which is separated from both half cystines by β -sheet A. Trp^{184} is located in a loop between the strand 3A in β -sheet A and helix F1, this loop is located almost the opposite pole to the two cystines. Trp^{267} included in helix H is separated from the two cystines by helix A. Thus, it is very likely that the circumstance of the three tryptophan residues, which are separated from the two cystines, is indistinguishable between the native and the reduced proteins. Although the contribution of the disulfide bond in the near-UV CD has not been established in ovalbumin, slight ellipticity difference observed at 260, 265 and 275 nm between the two forms of ovalbumin might be accounted for by a local conformational destabilization around some other aromatic side chains. Four tyrosines, Tyr^{106}, Tyr^{111}, Tyr^{117}, and Tyr^{125}, which appear to be close to the disulfide bond in the three dimensional structure (56), could be candidates for such aromatic residues.

In addition to the conformational data, the disulfide reduced ovalbumin was evaluated on its stability by differential scanning calorimetry (Koseki, unpublished data). The reduced ovalbumin was found to be less stable by $6.8^{O}C$ than disulfide-bonded one.

In conclusion, the disulfide bond in ovalbumin is not essential for its overall conformation, but affects, in a restricted extent, its local conformation around the two cystines that are involved in the disulfide bond in the native protein. Such local destabilization may be reflected in the decreased stability against heat denaturation as revealed by differential scanning calorimetry, although the free energy change for denaturation could not be quantitatively determined because of the lack of a reversible denaturation system in this protein (70,71). The present locally destabilized conformation should be differentiated from the 'Molten-globule'-like state that has been found with disulfide-bonded ovalbumin at acid pH (72), since at the extreme pH near-UV CD is almost completely diminished (72). In addition, no difference in intrinsic viscosity is observed between the native and disulfide-reduced ovalbumin (unpublished data). We, therefore, believe that the disulfide-reduced form of ovalbumin at neutral pH takes a native-like compact conformation.

Section 2

Refolding of reduced ovalbumin and
subsequent regeneration of intramolecular disulfide bond

Most of secretory proteins have intra-molecular disulfide bonds. The disulfide proteins can be classified into two groups ; one is including free sulfhydryl group and the other is not. In the most cases, the disulfide protein has only one free sulfhydryl group, if it has any (14). Exceptionally, ovalbumin has four free sulfhydryls in addition to one disulfide bond (64). Since this protein has additional free sulfhydryls, probability of an occurrence of mispaired disulfide bonds during the refolding process is increased. Whether the denatured ovalbumin could be refolded into the native conformation has been argued and there is no clear resolution. This controversy has been confused on the disulfide bond, since it was from before identification of the disulfide bond.

As shown in CHAPTER 2, the refolding of ovotransferrin, which has 15 intramolecular disulfide bonds, was achieved under the two step conditions; firstly, regaining of the native-like conformation, and secondly, regeneration of the correct disulfide bonds were proceeded. In the former section, it was confirmed that the ovalbumin under the non-denaturing conditions still has native-like conformation even on the reduction of the disulfide

bond.   If this disulfide-reduced ovalbumin were regarded as an

intermediate of the two-step refolding procedure, ovalbumin could

reach the complete refolding after reoxidization of the disulfide

bond.

In this section, the optimal conditions of the first-step

folding into the native-like conformation were searched and

subsequent regeneration of the correct disulfide bond   was

attempted   to form correct disulfide bond and its   native

conformation.

MATERIALS AND METHODS

Materials

Ovalbumin was purified from fresh egg white as described in

former section.   Dephosphorylated ovalbumin was prepared by acid

phosphatase treatment and ion-exchange chromatography   as

described elsewhere (19).   Trypsin treated with DPCC (type XI)

was purchased from Sigma.   The reduced form of ovalbumin was

prepared under non-denaturing conditions by incubating the native

protein at 1.0 mg/ml with 15 mM DTT[2] at 37°C for 2 hr in Buffer A

(50 mM Tris-HCl pH 8.2/1 mM Na$_2$EDTA).

CD measurement

The CD measurements for the native, the reduced, and the refolded ovalbumin were performed at protein concentration of 50 μ g/ml in 50 mM Tris-HCl buffer (pH 8.2) containing 1 mM Na_2 EDTA with a 1.0-mm cell. The CD spectra were measured in the wavelength from 200 nm to 250 nm. In order to monitor the refolding processes or the transition state to the equilibrium, the CD ellipticity was measured at fixed wavelength of 220 nm. The solution in the cell was kept at 25 $^{\circ}$C with a circulating water jacket attached to a cell holder.

Susceptibility to trypsin digestion

The susceptibility to trypsin was used as a probe for evaluating the conformation during the refolding of the protein. Ovalbumin was reduced and denatured at 20.0 mg/ml by an incubation with 3.5 mM DTT at 37 $^{\circ}$C for 30 min in Buffer B (8 M urea /50 mM Tris-HCl pH 8.2 /1 mM EDTA). Refolding of the reduced and denatured ovalbumin was conducted by 100-fold dilution with refolding buffer (50 mM Tris-HCl pH 8.2 /0.5 mM DTT) and incubation at 25 $^{\circ}$C. The aliquots drawn from the refolding ovalbumin solution were incubated at 0.2 mg/ml with various concentrations (0, 50, 100, and 500 μ g/ml) of trypsin at 25 $^{\circ}$C for 1 min. The digestion was stopped by the incubation at 0°C for 5 min with soybean trypsin inhibitor, the concentration

of which was twice of that of the protease. SDS-PAGE resoultion, staining with Coomassie blue, and densitometric analysis of the stained protein bands were performed as described in former section. In order to avoid the coagulation, the concentration of ovalbumin at diluted state was lowered in some experiments. In this series, the protein was reduced and denatured by an incubation with 5 mM DTT in Buffer B at 37^{0}C for 30 min. The reduced and denatured ovalbumin was diluted 20-fold by the refolding buffer to give the concentration of 50.0 μ g/ml. At the indicated period after dilution, the solution was incubated 50.0 μ g/ml with various concentrations (0, 12.5, and 125 μ g/ml) of trypsin at 25^{0}C for 1 min. Then, termination of the proteolysis and electrophoresis were conducted in the same way.

Detection of polimerized ovalbumin on native PAGE

The native ovalbumin was reuced and denatured at 20.0 mg/ml by an incubation with 3.5 mM DTT at 37 ^{0}C for 30 min in Buffer B. Refolding of the reduced and denatured ovalbumin was conducted by 100-fold dilution with refolding buffer (50 mM Tris-HCl pH 8.2 /0.5 mM DTT) and incubation at 25 ^{0}C for 2 hr. The refolding sample was applied to non-denaturing PAGE as descrived (26) with a slight modification. The PAGE gel prepared as described was equilibrated by pre-run until the voltage reach constant.

Additional pre-run with 20 mM cysteine containing electrophoresis
buffer for 15 min at constant voltage (100 V). Then, sample was
applied and electrophoresed with same buffer at same voltage for
4 hr. Staining of the gel was performed with Coomassie blue.

Disulfide bond analysis by two-step alkylation and urea PAGE

The reduced, denatured forms (1.0 mg/ml) of ovalbumin were
diluted 20-fold at $25^{\circ}C$ with Buffer A containing various
concentrations of GSH, and incubated at the same temperature for
3 hr. Reoxidization was initiated by the addition of GSSG
giving a distinct final concentration, and the mixture was
allowed to stand for 1 hr at the same temperature. Disulfide
bond in the protein was analyzed by a method described in CHAPTER
1.

Identification of disulfide bond

The regeneration of a disulfide bond after the denaturation
and reduction of disulfides and subsequent two-step refolding
procedure was examined by chemical analysis. The native
ovalbumin was reduced under denaturing conditions by an
incubation at 16.0 mg/ml with 5.0 mM DTT in Buffer A at 37 $^{\circ}C$ for
30 min. The thiol in this sample was diluted by a 16-fold
dilution with Buffer A containing 10 mM GSH. Then, in order to
reduce the denaturant (urea), subsequently it was 20-fold diluted

by Buffer B containing 10 mM GSH and incubated at 25 ^{O}C for 3hr. The regeneration of a disulfide bond was initiated by addition of 1 mM GSSG and the mixture was kept at same temperature for 1 hr. After that, the reoxidization was terminated by blocking of free sulfhydryls with 50 mM IAA for 5 min and subsequent addition of urea giving final concentration of 8 M. After 15 min incubation under co-existence of urea and IAA, 10 volume of cold acetone /N-HCl (98/2) was added to the mixture and the protein was precipitated by centrifugation (3,000 X g, 10 min) after leaving at -20 ^{O}C for 1hr, dissolved in Buffer B, and then separated from urea by gel-filtration using prepacked column (NAP-25, pharmasia-LKB-biotechnology) equilibrated with 3.6 mM Tris-HCl (pH 8.2). The urea removed sample was dried in vacuo and dissolved in Buffer B containing HCl giving pH 7.0 and heated to 80 ^{O}C for 10 min. After the heating, the sample was 4-fold diluted giving 2 M urea concentraton and digested at 1.6 mg/ml with 78.0 μ g/ml of DPCC-trypsin in the incubation at 30 ^{O}C for 6 hr. Termination of digestion was an addition of equal volume of 1 % trifluoloacetic acid solution.

The mixture (900 μ 1) was applied to a reverse phase HPLC column (YMC AP-302), and peptides were eluted with an acetonitrile linear gradient (0-80%) in 0.1% trifluoroacetic acid (pH 2.0). Peptide peaks were collected and 1/10 of each peak fraction was detected by Ellman method after reduction with

sodium borohydride were analyzed for their amino acid composition. The peaks exhibited DTNB sensitivity were further purified by reverse phase HPLC with an acetonitrile linear gradient (20-80%) in 10 mM triethylamine /acetic acid buffer (pH 5.0). The purified peptides were analyzed for amino acid compositions with an amino acid analyzer (Hitachi, model 835-30) and for primary sequences with an gas-phase protein sequencer (Applied Biosystems, model 477A/120A). Calculation of amino acid compositions was based on Alanine taken as indicated in parentheses.

RESULTS AND DISCUSSION

Effect of the protein concentration on refolding of the reduced ovalbumin

In order to proceed the refolding of ovalbumin according to the two-step procedure, the process of the first step in which the denaturant was diluted was investigated by far-UV CD measurement and resistance to trypsin. The refolding of the reduced and denatured ovalbumin was conducted by 100-fold dilution to give 0.2 mg/ml concentration at 25 $^{\circ}$C. As shown in fig. 17, compairing to the native ovalbumin, the CD ellipticity
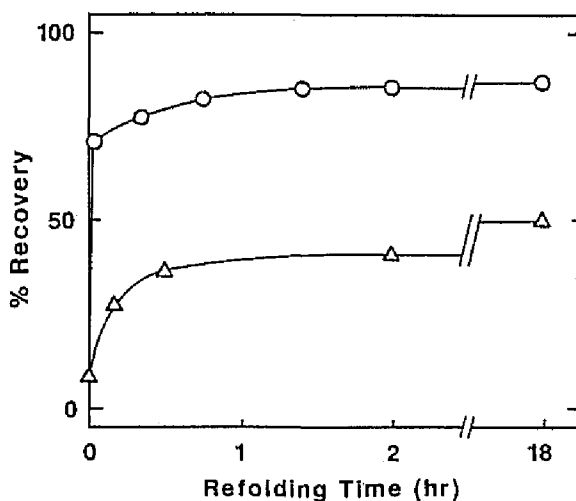
Fig. 17.   Recovery of the CD ellipticity and trypsin resistance
according to the refolding time.   Refolding was conducted to give
0.2 mg/ml of final protein concentration and it was monitored by CD
ellipticity at 220 nm (open circle) and the amount of the ovalbumin
molecule which was resistant to trypsin detected by SDS-PAGE (open
triangle).

at 220 nm was regained over 80 %, but the amount of the species

resistant to trypsin did not increased more than 50 % in 60 min.

This difference in the yield of refolding between the two

detection methods could be explained by the two possibility.

One is explained by an equilibrium of 50 % of the trypsin-

resistant ovalbumin and the rest 50 % of the trypsin-sensitive

species.   Another one is explained by the irreversible

transformaton of the reduced ovalbumin into the unknown species

which is trypsin-sensitive and has same CD ellipticity.   If it

is in a case, a distinct species different from the native and

the denatured ovalbumin can be detected by appropriate method.

If it is in a former case, trypsin-resistant ovalbumin would be reduced in the prolonged digestion period since the equilibrium would deviate from 50 % to compensate the transition of the equilibrium for the disappearance of the trypsin-sensitive one as a result of digestion.

(A)

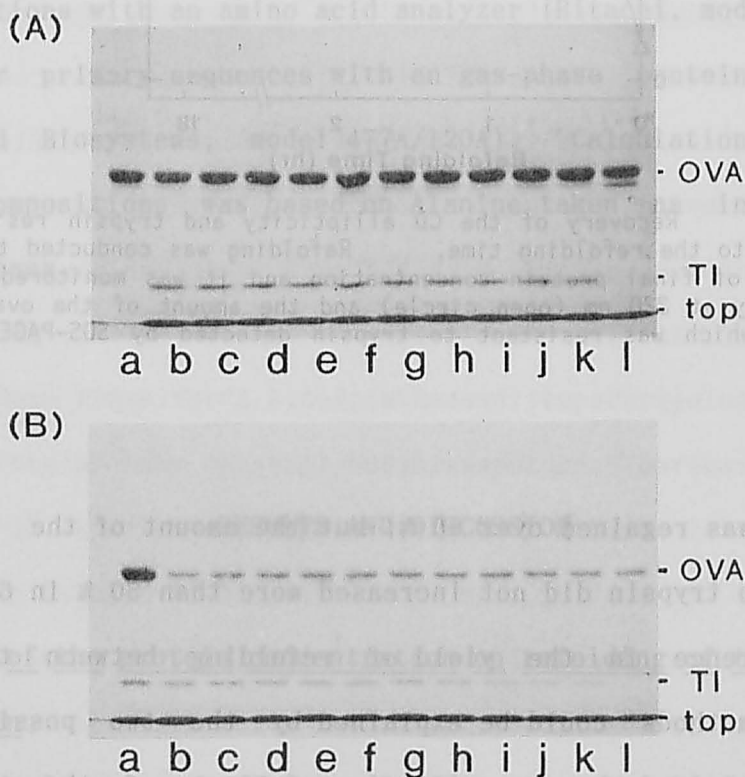a b c d e f g h i j k l

(B)

a b c d e f g h i j k l

Fig. 18.   Resistance to trypsin of ovalbumin in the prolonged reaction.   The disulfide-intact (panel A) and the 18 hr-refolded ovalbumin (panel B) was incubated at 0.2 mg/ml with 0.1 mg/ml of trypsin for 0 (lane b), 1 (lane c), 5 (lane d), 10 (lane e), 15 (lane f), 20 (lane g), 30 (lane h), 40 (lane i), 60 (lane j), 120 min (lane k), 18 hr (lane l).   The reaction was terminated by an incubation with soybean trypsin inhibitor.   Samples were resolved by SDS-PAGE and stained with Coomassie brilliant blue R-250.   The disulfide-intact ovalbumin (2.0 μg)(lane a, panel A) and the 18hr-refolded ovalbumin treated with the complex of trypsin and trypsin inhibitor preincubated at 0°C for 5 min (lane a, panel B) were applied for the control experiment.   Intact ovalbumin as OVA, the complex of trypsin and trypsin inhibitor as TI, and the top of migration as top are indicated on the right side.

Experimentally, fig. 18 shows the trypsin resistance in the prolonged reaction time more than 2 hr. Consequently, the possibility of the equilibrium between the trypsin-resistance and the trypsin-sensitive species was denied. Whether it is the case of another possibility was determined by searching for a distinct species different from both the native and the denatured protein. The reduced and denatured ovalbumin in 8 M urea buffer was diluted to 0.2 mg/ml and incubated at 25 $^\circ$C for 2 hr. That sample was electrophoresed under high pH, non-denaturing, and reducing conditions in order to detect the polymerized species.
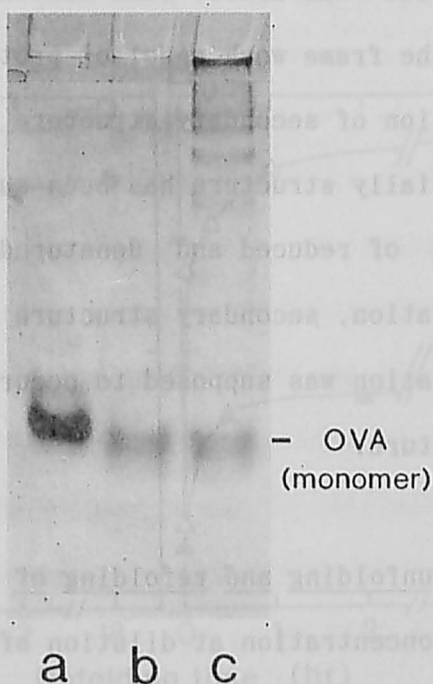

a   b   c

Fig. 19.   Detection of polymerized ovalbumin by native-PAGE under reducing conditions.   The 2 hr-refolded samples of ovalbumin (0.2 mg/ml) were applied to the native-PAGE under reducing conditions described in materials and methods with (lane b) or without (lane c) treatment with 0.05 mg/ml of trypsin at 25°C for 5min.   The native ovalbumin (3.0 μg)(lane a) was applied as a control experiment.

The reduced ovalbumin after dilution of urea was defined as mixture of the polymerized species and monomer ovalbumin in lane C of fig. 19. The same sample treated with trypsin before electrophoresis did not express any polymerized species in lane B besides the band corresponding to the monomer ovalbumin (lane A). Consequently, the poor yield of trypsin resistance on the dilution of urea was determined as a result of generation of the polymerized species which were degraded by trypsin. On the contrary to the poor resistance to trypsin, the CD ellipticity at 220 nm was recovered almost completely. This suggested the polymerized species has almost same contents of the secondary structure. In the frame work model of protein folding process, the quick formation of secondary structure and relatively slow formation of tertially structure has been supposed to occur (73). On the dilution of reduced and denatured ovalbumin at this protein concentration, secondary structure was evaluated to be formed and coagulation was supposed to occur before the formation of tertiary structure.

Reversibility of unfolding and refolding of the reduced ovalbumin

Since high concentration at dilution of denaturant resulted in poor recovery in trypsin-resistance, reduced ovalbumin diluted to lower concentration to achieve efficient refolding preventing coagulation. The reduced and denatured ovalbumin was diluted to

give 0.05 mg/ml concentration and investigated by far-UV CD measurement and resistance to trypsin. The concentration resulted in almost full recovery of trypsin resistance in 18 hr as well as CD ellipticity (fig. 20). In the following experiments, the final concentration of the refolding was stated at 0.05 mg/ml to avoid coagulation. The CD ellipticity was fully regained in its magnitude at 220 nm. Additionally, in order to evaluate the reduced ovalbumin after refolding, far-UV CD spectra were measured for the refolded, the reduced and the native ovalbumin. Figure 21 shows almost same spectra for three
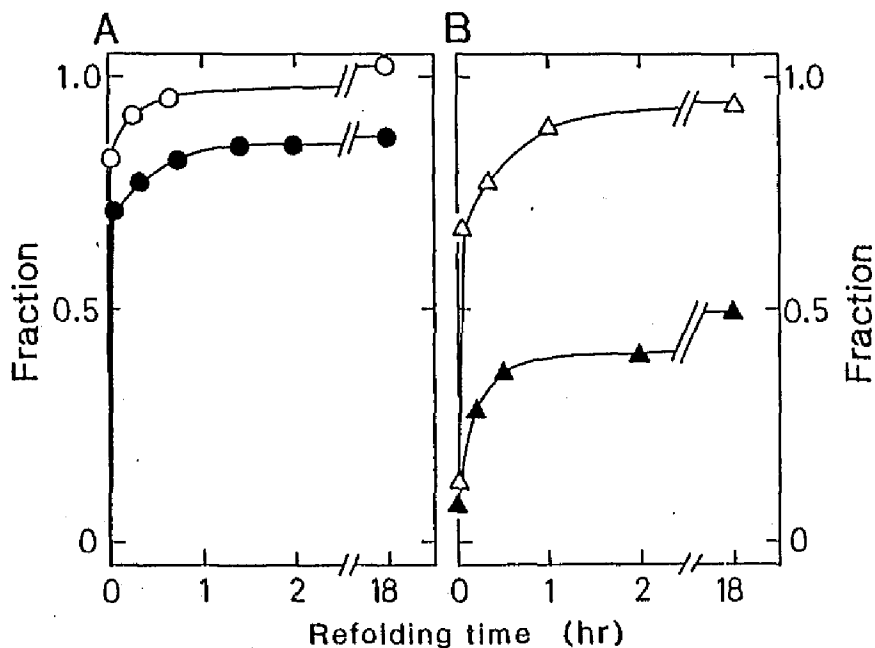
Fig. 20. Recovery of the CD ellipticity and trypsin resistance according to the refolding time at different protein concentrations. Refolding was conducted to give 0.2 mg/ml (closed symbols) or 0.05 mg/ml (open symbols) of final concentration. They were monitored by CD ellipticity (circles ; panel A) and the resistance to trypsin (triangles ; panel B) as in fig. 17.
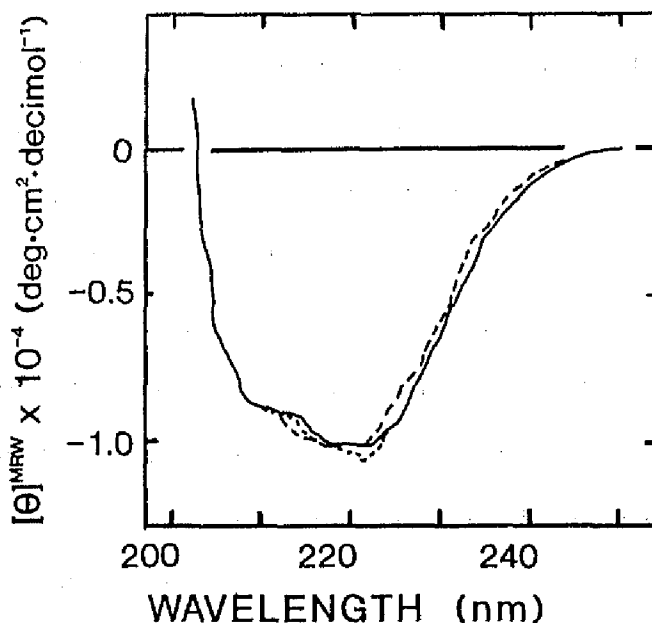
Fig. 21.   Far-UV CD spectra of the refolded, the native, and the
reduced ovalbumin.   The CD spectra of the 2 hr-refolded (slashed
line), the native (straight line), and reduced ovalbumin (dotted line)
were measured in a far-UV region (200 nm-250 nm).

species.   Thus, the refolded species was estimated to have   the

same   secondary   structure   contents as   the   native   one.   The

complete   resistance   to   trypsin (fig.   20)   also   revealed   the

tertiary structure of the refolded species to be the same as   the

native   one   also.   At the final protein concentration   of   0.05

mg/ml,   the reversibility of the refolding and the   unfolding   of

the reduced form of ovalbumin was investigated by measurement   of

CD   ellipticity at   220 nm on the equilibrium   at   various   urea

concentrations   (fig. 22).   Unfolding was initiated by   removing

the   reduced,   native-like   ovalbumin   into   distinct   urea

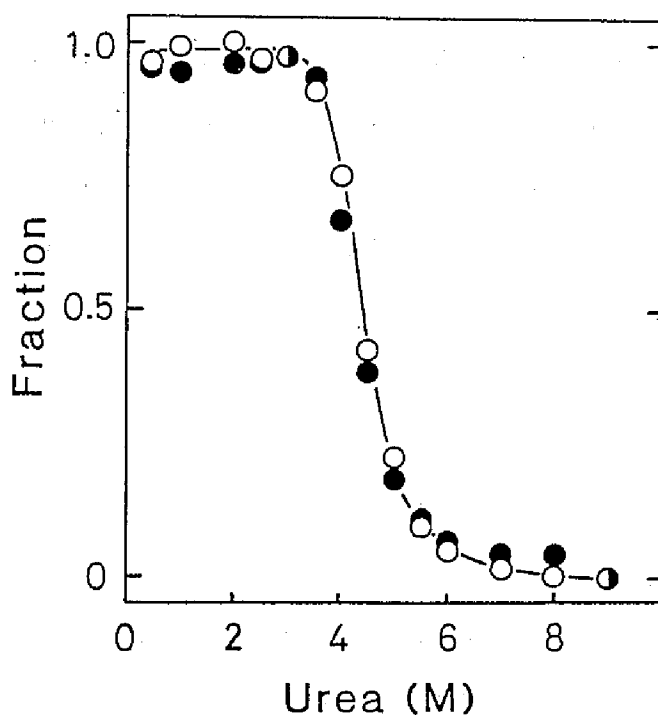concentration and refolding was initiated by removing   the

Fig. 22. Reversible folding process of the reduced ovalbumin. The CD ellipticity at 220 nm was measured for the reduced ovalbumin samples (0.05 mg/ml) in various concentrations of urea removed from Buffer A (open circles) or Buffer A containing 9 M urea (closed circles). The plotted data were the fraction values at their plato aginst the native one.

reduced, denatured ovalbumin into distinct urea concentration. Since the profiles of the refolding and the unfolding were coincident with each other, the alternate processes were estimated to be a reversible reaction and have no different intermediate. Although, the denaturation and the renaturation of ovalbumin have reported to be irreversible previously, this has been because the experiments were conducted without reduction of disulfide bond. Denaturation of protein with intramolecular

disulfide bond can cause random interconversion of sulfhydryl and disulfide form of half-cystine. On the renaturation of ovalbumin according to the two-step conditions described for ovotransferrin in CHAPTER 2, the first step folding of the reduced ovalbumin was confirmed to be accomplished at low protein concentration.

Redox conditions on reoxidization of the reduced ovalbumin from the refolded state

The reoxidization of the reduced disulfide bond was investigated as the final step to complete the renaturation of ovalbumin from the reduced and fully denatured state. For the initial state, the deduced native-like intermediate was prepared by dilution of the reduced and denatured ovalbumin. Reoxidization conditions was stated by the combination of the reduced form and oxidized form of glutathione as used in CHAPTER 2. The preferred ratio of the reduced and the oxidized

Fig. 23.   Formation of disulfide bond of ovalbumin under different redox conditions.   The reduced and denatured ovalbumin was diluted with buffer B containing 1 mM (panel A) or 10 mM (panel B) and incubated at 25°C for 3hr.   Reoxidization was initiated by adding of GSSG to give the final molar ratios to GSH (lanes b and c, 0; lanes d and e, 0.1; lanes f and g, 0.2; lanes h and i, 0.5; lanes j and k, 1.0).   It was terminated by blocking of free sulfhydryls with IAA in the absence of urea (lanes b, d, f, h, and j) and in the presence of 8 M urea (lanes c, e, g, i, and k).   Then, further treatment according to the two-step alkylation procedure was conducted as in CHAPTER 1. In lanes, a and l, the protein was alkylated by the one-step procedure with varying ratios of IAA to IAM.

A

B

0 2 4 6

a b c d e f g h i j k l

glutathione was saught by two-step alkylation and urea PAGE method. In the reoxidizing step, the concentrations of GSH and GSSG were varied and disulfide formation was estimated on the urea PAGE after two-step alkylation method. Fig. 23 shows disulfide bond formation and loss of reactivity with IAA according to concentration of GSSG at reoxidization step. When the refolding buffer contained 10 mM GSH and 1.0-5.0 mM GSSG, almost all ovalbumin was formed one disulfide bond in each molecule during 1 hr of the reoxidization. But in the presence of 1.0 mM GSH, significant amounts of the proteins with incomplete disulfide bonded molecule still remained at the same GSSG to GSH ratios. The disulfide bond formation has been proposed to be increased in the presence of higher concentration of GSH (74). The results in fig. 22 is, however, opposed to the idea. This could be caused by the high tendency to form scrambled disulfied bonds with reduced glutathione. As a result of the scrambled disulfides with glutathione, the species having four or less reactive sulfhydryl groups in neutral buffer containing 8 M urea were expressed after the two step alkylation at higher ratios of GSH / GSSG (1 / 1) in lane k on both panels A and B. Thus, the extra low reactivity in the denaturing conditions could be evaluated by the formation of scrambled disulfides with reduced glutathione. From these data, the concentrations of the reduced form and the oxidized form of

glutathione which were preferred to reoxidization of ovalbumin were determined 10 mM and 1 mM respectively.

Identification of the disulfide bond formation after refolding under the reducing conditions and subsequent reoxidization

According to the two-step refolding procedure and the redox conditions mentioned above, reoxidization of the refolded ovalbumin was conducted. The first step refolding of reduced and denatured ovalbumin was proceeded in the presence of 10 mM GSH at 25°C for 3 hr. After that, GSSG was added to the refolded ovalbumin giving 1 mM in the final solution. The reoxidization was performed by further incubation at 25°C for 1 hr and terminated by blocking of free sulfhydryl groups with IAA. It was investigated by identification of disulfide containing peptides from the tryptic digest of reoxidized sample, whether the reoxidized protein possessed the correct disulfide bond or not. The tryptic digest of the reoxidized ovalbumin was fractionated by reverse-phase HPLC on an ODS column with trifluoroacetic acid buffer at pH 2.0 (fig. 24). The six cystine containing peaks detected based on the Ellman method were analyzed for their amino acid composition. Only two peaks numbered 1 and 2 were evaluated to have disulfide bonds. They were further purified by rechromatography with same HPLC column in triethylamine / acetic acid buffer at pH 5.0 and analyzed for

Fig. 24.   Isolation of tryptic peptides containing disulfide bonds.
The reoxidized ovalbumin was digested with trypsin in the
presence of 2 M urea.   The digest was applied to a ODS coloumn (YMC
AP302) equilibrated with 0.1 % TFA.   Peptides were eluted with a
linear gradient of acetnitrile (0 to 80%)(slashed line).   The flow
rate was 1.0 ml/min.   Peptides were detected by measurement of an
absorption at 220 nm (upper straight line) and by a method using DTNB
(lower straight line).

Fig. 25.   Sequence analyses of the fractions containing disulfide
bond.   Purified fractions containing disulfide bond were analyzed
with protein sequencer.   The deduced fragments were expressed on the
primary structure around the intact disulfide bond (peak 1; surrounded
by solid line, peak 2; surrounded by dotted line).

Table 1.   amino acid compositions of tryptic peptides containing disulfide bond from reoxidized ovalbumin

Amino acid	peak 1	peak 2	Theoretical	
			A + B	A + B + C
Asx	2.81 (3)	3.89 (4)	3	3
Thr	0.93 (1)	1.41 (1)	1	1
Ser	3.01 (3)	4.19 (4)	4	4
Glx	3.66 (4)	5.52 (6)	4	6
Pro	2.49 (2)	3.28 (3)	3	3
Gly	2.61 (3)	3.73 (4)	3	3
Ala	– (1)	– (2)	1	2
(Cys	0.89 (1)	1.06 (1)	1	1)
Cys-O*	1.98 (2)	2.44 (2)	2	2
Val	2.46 (2)	3.86 (4)	3	3
Ile	1.86 (2)	2.48 (2)	2	2
Leu	3.44 (3)	4.76 (5)	4	5
Tyr	1.54 (2)	1.49 (1)	2	3
Phe	1.68 (2)	2.23 (2)	2	2
Lys	1.72 (2)	2.35 (2)	2	2
His	0.71 (1)	0.97 (1)	1	1
Arg	0.87 (1)	1.30 (1)	1	2
	32.77	45.88	38	44

* cysteic acid determined after oxidation with performic acid

sequence A (Phe[59]-Arg[84]); FDKLPGFGDSIEAQCGTSVNVHSSLR
sequence B (Tyr[111]-Lys[122]); YPILPEYLQCVK
sequence C (Leu[105]-Arg[110]); LYAEER

their N-terminal amino acid sequence.   Each peak had two fragments and they were determined in reference to the primary structure of ovalbumin.   From the deduced tryptic peptides, two candidates were selected for each peak (fig. 25).   The results of the amino acid analysis supported the deduction of amino acid sequences of the fragments (table 1).   The two peaks proved to have one disulfide bond between Cys^{73} and Cys^{120}.   That is an original disulfide bond which is in the native ovalbumin molecule.   As the results of the amino acid analysis after a selection by a method using DTNB, no other disulfide-containing peptide was detected.   From these data, the author concluded that the regeneration of a correct disulfide bond from the reduced and fully denatured state was attained.

In conclusion, ovalbumin : a model of proten containing both the disulfide bond and the free sulfhydryl groups, was able to be regained the native conformation and the correct disulfide bond from fully denatured state.   This was achieved according to the two-step conditions, which include the primary folding in the reducing buffer and the subsequent reoxidization.

SUMMARY

In this thesis, it was investigated whether the conformation of a protein could be constructed from its primary structure. Folding of disulfide proteins with relatively high molecular weight has been thought to be difficult. Thus, on the two disulfide proteins from egg white, optimal conditions for renaturation were searched.

In CHAPTER 1, a specific, sensitive, and clear method to evaluate the renaturation processes was developed. It included two-step alkylation and PAGE, which enables the estimation of protein species with different number of free sulfhydryls distinctly.

In CHAPTER 2, an egg white protein with 15 disulfide bonds ; ovotransferrin was attempted to renature. Previous simple method for reoxidization was proved to be useless for the protein with many disulfides. Alternatively, a two-step procedure was found to be useful for the efficient refolding of a complex protein. In the first step of reducing and low temperature conditions, the reduced and denatured protein could fold into native-like structure. In the subsequent second step, oxidized glutathione enhanced the formation of correct disulfide bonds. The intermediate state of reduced and native-like molecule was suggested to be important for the effective renaturation.

In CHAPTER 3, another egg white protein with one disulfide bond and four free sulfhydryl groups ; ovalbumin was attempted to renature according to the two-step procedure described in CHAPTER 2. Disulfide-reduced form of ovalbumin was investigated on its conformation as a model of refolding intermediate in section 1. Near-UV CD analysis, susceptibility to subtilisin, and reactivity of the sulfhydryl groups revealed local conformational change on reduction of the disulfide bond of native ovalbumin. On the contrary, its overall structure was confirmed to be native-like even on reduction of disulfide bond as the results of far-UV CD measurement and susceptibility to trypsin. In order to complete the renaturation of ovalbumin, the first step refolding and the second step reoxidization were putted into practice in section 2. The refolding step was proved to be completely reversible, though it has been regarded as irreversible process. As a result of the next reoxidization, only correct disulfide bond was determined. According to the two-step refolding procedure, correct renaturation was confirmed for ovalbumin.

In conclusion, since the complex disulfide proteins such as ovotransferrin and ovalbumin could get efficient renaturation according to the two-step procedure, the conformation of protein proved to be able to regain on the basis of its primary structure.

ACKNOWLEDGMENTS

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