Denatured State of Ovalbumin in High Concentrations of Urea as Evaluated by Disulfide Rearrangement Analysis*

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To investigate the highly denatured state of ovalbumin (molecular mass of 42.7 kDa, four cysteine sulfhydryls and one cystine disulfide) using the disulfide rearrangement approach, we established the peptide-mapping procedure using a cysteine-labeling technique with a fluorescent dye that allows the quantitative analyses for the disulfide-involved half-cystines. Ovalbumin denatured at a low protein concentration in 8–10 M urea, in which the protein showed complete unfolding as evaluated by far-UV CD spectra, was analyzed for the disulfide-involved half-cystines using the peptide-mapping procedure. Data clearly showed that the number of free sulfhydryls and intrachain disulfides were four and one, respectively, but that all six half-cystines are labeled with the dye. These results strongly suggested that 15 disulfide isomers that are theoretically possible for a molecule having one disulfide and four sulfhydryls are all generated during the denaturation. The quantitative data for the ratios of the observed labeling values for the six half-cystines, relative to the overall labeling values, were consistent with the view that the distribution of the 15 possible disulfide isomers at equilibrium depends on the number of amino acid residues separating the two half-cystines to the power of −1.9 to −2.0. Essentially, the same non-gaussian chain nature was also observed with kinetic data for sulfhydryl-disulfide exchanges after denaturation of native ovalbumin.

For the investigation of conformational distribution, the sulfhydryl-disulfide rearrangement problem may provide a unique model system. It has been known that disulfide rearrangements take place intramolecularly in denatured states, giving many disulfide isomers with non-native disulfide bonds (Creighton, 1984). If a protein that contains multiple sulfhydryls and a disulfide in a molecule is in an ideal random-coil state, every sulfhydryl will be able to freely encounter a disulfide, thereby all possible disulfide isomers are generated. Such a disulfide isomer corresponds to a subset consisting of a large number of conformational isomers, and an entropically favorable disulfide isomer that includes the greatest number of conformational isomers dominates over the distribution. Early statistical calculations have shown that the probability of the occurrence of such disulfide isomers is proportional to $n^{-3}$, where $n$ is the number of amino acid residues separating the two half-cystines that are involved in a disulfide bond (Kauzmann, 1959; Poland and Scheraga, 1965). Chan and Dill (1990, 1991) have recently pointed out that, unlike such Gaussian distributions, the probability may be more consistent with a dependence upon $n^{-3.4}$ for random coil polymers with significant excluded volume effects. Few attempts, however, have been made to experimentally investigate the feasibility of the polymer chain theories for the highly denatured state of a protein.

As a useful model system, we have been interested in the disulfide rearrangement problem of ovalbumin. The egg white protein consists of a single polypeptide chain of 365 amino acid residues, and contains six half-cystines of SH-1 (Cys-111, SH-2 (Cys-30), SH-3 (Cys-73), SH-4 (Cys-120), SH-5 (Cys-367), and SH-6 (Cys-382). In the native state, SH-3 and SH-4 form a disulfide bond, and the other four occur as free cysteine sulfhydryls (Thompson and Fisher, 1978; Niabet et al., 1981). Our indirect evidence has, however, shown that some non-native disulfide bonds may be produced in this large disulfide protein in a high concentration of urea (Takahashi and Hirose, 1992). In this protein, the six half-cystines are widely allocated from the N-terminal to the C-terminal region, indicating that disulfide rearrangement reactions will occur in the overall molecule. In the present report, we have established a peptide-mapping procedure that allows the quantitative analysis for the ratios of disulfide-involved half-cystines that are directly related to the distribution of the 15 disulfide isomers. Using this peptide-mapping procedure, sulfhydryl-disulfide exchanges in highly denatured ovalbumin were analyzed kinetically and at the equilibrium state. On the basis of the exchange data, it was investigated whether or not ovalbumin behaves as a Gaussian chain in its highly denatured state.

EXPERIMENTAL PROCEDURES

Materials—Ovalbumin was purified from fresh egg white by crystallization in an ammonium sulfate solution and recrystallized five times (Sørensen and Hayrup, 1915). From the crystallized ovalbumin, A$_2$-ovalbumin (diphosphorylated form) was purified by ion-exchange chromatography in the same way as described by Kitabatake et al. (1990),

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nylcarbamyl chloride (type XI) and chymotrypsin (type II) were purchased from Sigma. *Achromobacter* protease I (EC 3.4.21.50) was obtained from Wako Pure Chemical Industries. Urea was specially prepared reagent grade and other chemicals were guaranteed grade from Nacalai Tesque.

**CD Analyses for Denaturation Transition—**Ovalbumin was incubated at 0.2 mg/ml and 37 °C in TE buffer (50 mM Tris-HCl, 1.0 mM EDTA, pH 8.8) containing different concentrations of urea. CD ellipticity at 222 nm was determined at various time intervals with a spectropolarimeter (JASCO, J-700) using a 0.1-cm cuvette.

*Polyacrylamide Gel Electrophoresis—*The number of intrachain disulfide bonds in ovalbumin was determined by selective two-step alklylation and subsequent PAGE as described previously (Takahashi and Hirose, 1990). Briefly, ovalbumin denatured in TE buffer containing 9 M urea was alkylated as the first step with 50 mM iodoacetic acid, precipitated with cold acetone, 1 N HC1 (98:2), washed three times with acetone, 1 N HCl, H2O (98:2:10), dissolved in TE buffer containing 9 M urea, reduced by 12 mM DTT, and then alkylated by 35 mM iodoacetamide as the second step. The protein was electrophoresed on a polyacrylamide gel using a high pH urea-denaturing buffer and stained with Coomassie Blue.

The protein sample that had been alkylated in the first step with iodoacetic acid was also analyzed by non-reducing SDS-PAGE; the alkylated sample was mixed with a 0.33 volume of 0.2 M Blue.

**Peptide Mapping—**As a prerequisite for the determination of half-cystines involved in disulfide bonds, all six half-cystines were labeled with a fluorescent alklylation reagent, IAM, and subjected to the peptide mapping analysis. Ovalbumin was incubated at 0.2 mg/ml in TE buffer containing 5 mM DTT and 8 M urea at 37 °C for 30 min. The free sulfhydryls in the disulfide-reduced, denatured protein were labeled by incubation at 37 °C for 10 min with 15 mM IAEDANS in TE buffer containing 8 M urea. Excess IAEDANS was trapped by incubation with 18 mM DTT at 37 °C for 5 min. The mixture, 10 volumes of cold acetone, 1 N HCl (98:2) solution was added, and then proteins were precipitated by centrifugation (3000 x g, 5 min). After the precipitates were washed three times with cold acetone, 1 N HCl, H2O (98:2:10), the labeled ovalbumin was dissolved in TE buffer containing 8 M urea giving a protein concentration of 2 mg/ml, fully denatured by incubation at 30 °C for 60 min, and diluted 4-fold with TE buffer giving 2 mg/ml ovalbumin.

The protein was extensively digested in a two-step manner: in the first step with 20 units/ml trypsin and 1 unit/ml *Achromobacter* protease I and 1 unit/ml chymotrypsin at 30 °C for 20 h; in the second step with an additional 20 units/ml *Achromobacter* protease I and 1 unit/ml chymotrypsin at 30 °C for 20 h. To obtain reproducible digestion, prior to the ovalbumin digestion, we assayed the protease activities as described by Walsh and Wilcox (1970) using synthetic substrates: *p*-toluenesulfonyl-1-arginine methyl ester for trypsin activity, N-benzyloxyl-1-lysine ethyl ester for chymotrypsin activity and *p*-toluenesulfonyl-1-lysine methyl ester for *Achromobacter* protease I activity. One unit was defined as an enzyme activity that hydrolyzed 1 pmol of substrate per min at 30 °C.

The protein digestion was terminated by the addition of a 0.1 volume of 5% trifluoroacetic acid solution. The mixture corresponding to 0.25 mg of the original ovalbumin was applied to a reverse phase HPLC column (Cosmosil 5C4-AR-300: ODS, 4.6 x 150 mm) connected to a HPLC apparatus (Shimadzu, LC-10A). Peptides were eluted with an acetonitrile linear gradient in 0.1% trifluoroacetic acid. Cysteine peptides were analyzed using a synchrotron radiation source and for primary sequences with a gas-phase protein sequenator (Applikon Biotechnol, model 477A/120A). For the amino acid analysis, the peptides were hydrolyzed in the gas phase with 6 N hydrochloric acid containing 0.1% (v/v) phenol for 24 h at 110 °C under vacuum.

**Protease digestion**

![HPLC](image)

**Fig. 1. Schematic representation for the determination of cysteine peptides labeled with a fluorescent dye.** Ovalbumin has one intramolecular disulfide bond and four sulfhydryls. In this figure, the case for protein species with a non-native disulfide bond (S-S) consisting of SH-5 and SH-6 is shown as an example. Protein sulfhydryls were first quenched in acid-urea solution. In the sample run, the four cysteine sulfhydryls (SH) were trapped with a high concentration of iodoacetamide (IAM) yielding alkylated cysteines (SM). The labeled disulfide was then reduced by DTT, and newly generated sulfhydryls were labeled with a fluorescent alklylation reagent, IAEDANS giving IAEDANS-labeled cysteines. The labeled protein was extensively proteolyzed and fractionated by HPLC. In the standard run, the first alklylation step was skipped, thereby all six half-cystines were allowed to be labeled with the fluorescent dye. Fluorescence peak areas corresponding to cysteine peptides were determined, and the relative ratios, R_, R_, . . . , or 6 of disulfide-involved cysteines were determined using Equation 1. In this example, either R_ or R_ should be 1.0, whereas R_ should be zero. When the original ovalbumin consists of the 15 possible disulfide isomers, R_ should distribute in all six half-cystines depending on the distribution of the isomers, but the sum of R_ should be 2.0 regardless of the state of original ovalbumin.

The abbreviations used are: HPLC, high performance liquid chromatography; IAM, iodoacetamide; IAEDANS-labeled, N-acetyl-N'-5-sulf-1-naphthylethylamide; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
with 15 mm IAEDANS. For the "standard," the acid-quenched protein was mixed with the same 1.6 volume of 0.3 M Tris-base containing 9.5 M urea and 5 mm DTT, incubated at 37 °C for 30 min, precipitated in acetic-HCl, dissolved in TE-urea buffer, and labeled with IAEDANS in the same way. For the "blank" (not depicted in Fig. 1), the acid-quenched protein was mixed with the same Tris-base containing 5 mm DTT and urea, incubated at 37 °C for 30 min, alkylated with 0.2 M iodoacetamide, precipitated in acetic-HCl, and then labeled with IAEDANS in the same way.

The AEDANS-labeled proteins were precipitated in acetic-HCl, extensively proteolyzed in the two-step way, and analyzed by reverse-phase HPLC in the same way as in the peptide-mapping procedure. In this HPLC analysis, the mixture corresponding to 0.05 mg of original ovalbumin was analyzed, since fluorescence peak areas in the standard run were linear up to 0.25 mg of original ovalbumin. We confirmed that in the blank run, no AEDANS-labeled peaks were detected for all six half-cystines. For each of the six half-cystines, SHR (i = 1, 2, . . . , or 6), fluorescence peak areas in the sample and standard runs were determined. In the HPLC, either SH-1 (Cys-11), SH-5 (Cys-367), or SH-6 (Cys-382) was eluted as a single peak, but SH-2 (Cys-30), SH-3 (Cys-73), or SH-4 (Cys-120) was separated into two peaks (see Figs. 2 and 3). Thus, the sums of two peak areas for SH-2, SH-3, and SH-4 were determined. The occurrence of only one intramolecular disulfide was unchanged in either the native or denatured state (see Fig. 4), indicating that two labeled half-cystines existed all the time in an ovalbumin molecule. Thus, the ratio of a disulfide-involved half-cystine (\( R_{SH,i} \), i = 1, 2, . . . or 6) to the total of 2.0 was determined using the following equation:

\[
R_{SH,i} = 2 \times (A_{SH,i}/B_{SH,i}) \sum_{i} (A_{SH,i}/B_{SH,i})
\]

where \( A_{SH,i} \) and \( B_{SH,i} \) represent the peak areas for AEDANS-labeled half-cystine in the sample and standard runs, respectively, with respect to one of the half-cystines, \( SH, i \).

**Fitting the Data**—The theoretical value for the ratio of a labeled half-cystine, \( R_{SH,i} \), was calculated on the basis of the assumption that the distribution of 15 disulfide isomers at equilibrium of the sulfhydryl-disulfide exchanges depends on loop length to a power of \( p \). To estimate a suitable value for \( p \), we calculated the sums of squares of the deviations of the observed data, \( R_{SH,i} \), from the theoretical one, \( R_{SH,i} \),

\[
\sum_{i} (R_{SH,i} - R_{SH,i})^2
\]

at varying \( p \) (ranged from 1 to 3 in 0.01 steps) values and searched for a \( p \) value giving a minimum deviation (see Fig. 5).

By searching for the best fitting value of \( p \) by kinetic analyses, the time course curves calculated on the basis of a model shown in Fig. 7 were fitted to the observed \( R_{SH,i} \) at various denaturation times (see Fig. 6) by using a program written in this laboratory that utilizes a Levenberg-Marquardt algorithm (Marquardt, 1963) and a Runge-Kutta algorithm in combination.

**RESULTS AND DISCUSSION**

**Peptide Mapping for Labeled Cysteines**—To establish the peptide mapping for the six half-cystines, the disulfide-reduced ovalbumin was labeled with IAEDANS, extensively digested with proteases, and fractionated by reverse-phase HPLC. As shown in Fig. 2, many fluorescent peaks were detected. Amino acid analyses for 20 major peaks revealed that only nine peaks denoted A to I are cysteine peptides, and that the others are non-peptide substances or some non-cysteine peptides. All nine peaks labeled A to I were further fractionated by rechromatography on the same HPLC column using a different buffer system. The purified cysteine peptides were analyzed for their amino acid sequences from the N-terminal to C-terminal with a sequenator. According to the established sequence of ovalbumin (Nisbet et al., 1981), the nine peptides were assigned as shown in Fig. 3. The data for amino acid analyses were consistent with the sequence; in all nine peptides, the occurrence of one carboxymethyl cysteine that was the acid-hydrolysis product of the AEDANS-labeled cysteine was confirmed (data not shown).

It is generally observed that a large peptide is poorly recovered on reverse-phase HPLC. This may interfere with reproducible peptide quantifications by HPLC. The proteolysis conditions employed here that included extensive digestion with the three different proteases in combination generated small peptides consisting of up to eight amino acids with quite high recoveries of at least 60.5% (SH-3: 19.4 ± 41.1%) from the original albumin (Fig. 3). We confirmed that the peptide recoveries are closely correlated (correlation coefficient, 0.930) with the relative fluorescence peak areas for the corresponding cysteine-peak. In addition, the ratios of the fluorescence peak areas for the six half-cystines in the standard run were highly reproducible. We, therefore, concluded that the present peptide mapping procedure is suitable for the quantitative analysis for disulfide-involved half-cystines in ovalbumin.

**Denaturation Transition and the Number of Intraehain Disulfide Bond**—Using far-UV CD-analyses, we searched for conditions that induced the fully denatured state in ovalbumin. Ovalbumin was incubated with various concentrations of urea.
Denatured State of Ovalbumin in Denaturant

Mobility of ovalbumin was fully reduced by DTT, alkylated with iodoacetamide as the first step. In Panel A, the alkylated ovalbumin was fully reduced by DTT, alkylated with iodoacetamide as the second step, and then analyzed by high pH PAGE as described in the text. The standard protein for the calibration of the introduced number of iodoacetic acid was prepared as described before (Takahashi and Hirose, 1990). The numbers on the left side indicate the introduced number of the negative charges of iodoacetic acid. In Panel B, the mobility for the disulfide-bonded and disulfide-reduced forms of the data at apparent equilibration were recorded. Denaturation transition was observed at urea concentrations of 4.5–5.5 M. At urea concentrations higher than 8 M, the protein appeared to be almost completely denatured.

It is a prerequisite for the evaluation of denatured ovalbumin by the disulfide rearrangement analysis that the number of intrachain disulfide bonds is maintained at one under all denaturation conditions. Conventional analysis for protein sulfhydryls and disulfides, such as the optical technique using dithiobisnitrobenzoic acid or amino acid analysis after cysteine alklylation provides only information about an average number per protein molecules. We, therefore, employed an alternative method which includes the two-step alklylation and subsequent PAGE, since protein species with different numbers of disulfide bonds can be distinctly detected by this technique (Takahashi and Hirose, 1990). Ovalbumin was incubated in 9 M urea for various times, and the protein disulfide was analyzed using the PAGE technique. As shown in Fig. 4A, the number of disulfide bonds per protein molecule was one during a denaturation time as long as 2 h.

As shown in Fig. 4B, in non-reducing SDS-PAGE, protein mobility was slightly changed during the denaturation. The mobility of ovalbumin was, however, clearly greater than that of glutamate dehydrogenase (molecular mass, 55.6 kDa), indicating that an oligomer due to interchain disulfide bonds was not formed. We, therefore, concluded that the sulfhydryl-disulfide exchange, if it occurs, should be intramolecular under the employed denaturation conditions, and that no additional intramolecular disulfides are formed. The alteration of protein mobility in non-reducing SDS-PAGE during denaturation may be related to the accumulation of conformational isomers with short disulfide loops (see Fig. 6), since the mobility of the protein species generated during denaturation was very similar to that of disulfide-reduced ovalbumin (Fig. 4B).

Quantification of Disulfide-involved Half-cystines at Equilibrium of Sulphydryl-Disulfide Exchanges—Ovalbumin was denatured at 37 °C and pH 8.8 in 9 M urea, in which the protein was almost completely denatured as evaluated by CD ellipticity, and the ratio of AEDANS-labeled half-cystine ($R_{SH,i}$) was determined at various denaturation times of 0–4 h by the peptide-mapping analysis. The data showed that exchange reactions in 9 M urea reached an equilibrium after a 2-h incubation. The $R_{SH,i}$ values at 2-h denaturation in different urea concentrations of 8 and 10 M that also induced complete denaturation as evaluated by CD ellipticity were essentially the same as those in 9 M urea ($R_{SH,i}$ values for SH-1, SH-2, SH-3, SH-4, SH-5, and SH-6, respectively: 0.286, 0.353, 0.198, 0.138, 0.499, and 0.522 in 8 M urea; 0.294, 0.355, 0.177, 0.129, 0.510, 0.533, in 10 M urea).

That these $R_{SH,i}$ values correctly reflected the inherent distribution of the disulfide isomers in highly denatured ovalbumin was supported by the following observations: 1) the labeled half-cystines were restricted to SH-3 (Cys-73) and SH-4 (Cys-120), native ovalbumin was analyzed under the alklylation conditions employed here (see the data for denaturation at time 0 in Fig. 6). Thus, it is very unlikely that some sulfhydryl-disulfide rearrangement reactions during the alklylation (Weissman and Kim, 1991) affect the inherent distribution of the disulfide isomers. 2) The $R_{SH,i}$ values in 9 M urea at a pH of 9.3 were almost exactly the same as those at pH 8.8 for all six half-cystines (data not shown). This excludes the possibility that the six free sulfhydryls in the denatured state have significantly different $p\kappa$ values in such a way that the inherent distribution of disulfide isomers are greatly affected by changing pH conditions (Darby and Creighton, 1993).

Previous theoretical conclusions show that the probability of the occurrence of disulfide isomers depends on loop length (the number of amino acid residues separating the two half-cystines) to a power of $–p$; results from statistical calculations (Kauzmann, 1958; Poland and Scheraga, 1965) indicate a relation of $p = 1.5$, while a lattice model theory is consistent with a relation of $p = 2.4$ (Chan and Dill, 1990, 1991). We examined how the observed ratios of labeled half-cystines at equilibrium are related to the theoretical conclusions. In the case of the lattice model, the $R_{SH,i}$ values for six half-cystines (SH-i) can be calculated as follows: 0.344 for SH-1 (Cys-11), 0.373 for SH-2 (Cys-30), 0.101 for SH-3 (Cys-73), 0.0501 for SH-4 (Cys-73), 0.566 for SH-5 (Cys-367), and 0.566 for SH-6 (Cys-382) for a total of 2.0. According to the statistical calculations, the ratios of labeled half-cystines can be predicted to be 0.369 for SH-1, 0.412 for SH-2, 0.219 for SH-3, 0.136 for SH-4, 0.432 for SH-5, and 0.431 for SH-6. We estimated the deviations of the observed $R_{SH,i}$ values from the predicted ones, $\hat{R}_{SH,i}$. For three varying urea concentrations (8, 9, and 10 M urea), the deviations were from 0.0223 to 0.0314 for the statistical theory and from 0.0214 to 0.0272 for the lattice model theory. These very

similar deviation data made it difficult to answer the question of which theory is more feasible for the elucidation of the highly denatured state of ovalbumin. We, therefore, searched for the \( p \) values that give the minimum deviations. As shown in Fig. 5, the deviations were minimized at a \( p \) value that is intermediate between the two extremes of 1.5 and 2.4; the deviations were minimized at \( p \) values of 1.87, 1.99, and 1.89 in 8, 9, and 10 M urea, respectively.

**Kinetic Analysis for Sulphydryl-Disulfide Exchanges—Time course for the sulphydryl-disulfide exchange in highly denatured ovalbumin was investigated by quantifying the disulfide-involved half-cystines.** As shown in Fig. 6, the ratios of labeled half-cystines for SH-3 (Cys-73) and SH-4 (Cys-1201), which form a disulfide in the native state, were rapidly decreased with denaturation time. Most of the native disulfide appeared to be first transferred to a disulfide consisting of SH-1 and SH-2, since the decreases in labeled SH-3 and SH-4 appeared to be compensated by the increases in labeled SH-1 and SH-2 for a denaturation time as short as 10 min. Greater accumulation at the 10 min denaturation of labeled SH-1 (Cys-11) and SH-2 (Cys-30) than labeled SH-3 (Cys-73) and SH-4 (Cys-120) may be accounted for by the fact that the former two half-cystines can form a much shorter disulfide loop than the latter two. These four labeled half-cystines then gradually decreased, while labeled SH-5 and SH-6 increased at a slow rate. These slow increases compared to those for SH-1 and SH-2 may reflect the fact that SH-5 is localized at a farther site from SH-4 in the primary structure than SH-2 from SH-3.

Fitting of the kinetic data in Fig. 6 was carried out on the basis of the model shown in Fig. 7. In this model, the native protein, N[3-4] is first transformed into the fully denatured form having the native disulfide, D[3-4] in which the exchange reaction is initiated. The sulphydryl-disulfide exchange reactions in the first step generate eight non-native disulfide isomers; in the second step of the exchanges, all 15 disulfide isomers are produced (Fig. 7A). Four free sulphydryls in every isomer can contact a disulfide, thereby 60 different exchange reactions are included in the interconversions among 15 disulfide isomers. The rate constant for an exchange reaction should be proportional to the probability for such contact. According to Kauzmann (1959), the probability depends on \( n_{eq}^{2} \), where \( n_{eq} \) is the “effective number” of amino acids separating a disulfide and a relevant sulphydryl. The value of \( n_{eq} \) corresponds to \( n_{e} \),
quite rapid with a first-order rate constant of 0.264 min\(^{-1}\). Using this rate for the reaction from N(3-4) to D(3-4), the observed data for labeled half-cystines in Fig. 6 were fitted to the model and \(m\) and \(p\) were allowed to vary. The best fit curves shown in Fig. 6 gave \(k = 820 \text{ n}_\text{m}^{-1}\). The value for \(p\) (1.92) was again an intermediate between the two extremes of 1.5 and 2.4, and was almost exactly the same as the data obtained for the equilibrium analysis in 9 M urea. In a previous report, Darby and Creighton (1993) have pointed out that the rates for disulfide formation in bovine pancreatic trypsin inhibitor in the presence of urea depends on \(n\) (the number of amino acids separating two half-cystines) more suitably to a power of \(-2.4\) than to \(-1.5\). Our best-fit calculation for their data, however, are consistent with 1.84 for the \(p\) value. The intermediate values for \(p\) may be therefore common to the urea-denatured states of ovalbumin and bovine pancreatic trypsin inhibitor.

CONCLUSIONS

Unlike conventional methods for protein analysis, the disulfide-rearrangement approach employed in the present study was found to provide unique information about the denatured state of a protein; some information about the distribution of conformational isomers by an equilibrium analysis and about the dynamic chain nature by a kinetic approach. Both equilibrium and kinetic data for highly denatured ovalbumin revealed that all cysteine sulfhydryls in this large protein can freely encounter a disulfide bond. In addition, the greatest ratios of labeled half-cystines were detected with SH-5 and SH-6, which can form the entropically favored shortest disulfide loop. We, therefore, qualitatively conclude that ovalbumin essentially behaves as a random coil polymer in high concentrations of urea.

More quantitatively, the previous theoretical conclusions demonstrate that the probability of the occurrence of disulfide isomers depends on loop length to a power of \(-p\); the \(p\) value is 1.5 for an ideal Gaussian chain (Kauzmann, 1959; Poland and Scheraga, 1965), but it is estimated to be 2.4 for a self-avoiding chain with significant exclusion volume (Chan and Dill, 1990, 1991). The \(p\) value is a highly important factor for the prediction of the effect of a disulfide bond on the conformational entropy in a protein: previous estimations have all based on the Gaussian behavior for disulfide proteins (Lin et al., 1984; Ueda et al., 1985; Goto et al., 1987; Pace et al., 1988). Both the equilibrium and kinetic data from urea-denatured ovalbumin were, however, consistent with a \(p\) value from 1.9 to 2.0. Highly denatured ovalbumin, therefore, appears to behave as an intermediate between the two extremes of the ideal Gaussian chain and the self-avoiding chain. The present experimental data, however, are restricted to the protein species of ovalbumin and the denaturing conditions in high concentrations of urea. Also, the previous theoretical models (Kauzmann, 1959; Poland and Scheraga, 1965; Chan and Dill, 1990, 1991) depend on the calculation using a model polymer consisting of homologous monomers. Extensive examinations of a variety of disulfide proteins under various denaturing conditions and introduction of a theoretical model for a protein-like heteropolymer would help better understand the highly denatured states of proteins.

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REFERENCES

Poland, D. C., and Scheraga, H. A. (1965) Biopolymers 3, 379-399