Studies on the Relationship of Disulfide Bonds to the Formation and Maintenance of Secondary Structure in Chicken Egg White Lysozyme†

Frederick H. White, Jr.

ABSTRACT: The dependence of secondary structure on SS content was examined to gain further insight into the role of SS bond formation in chain folding. Lysozyme was reduced to various levels, and the products were studied with respect to remaining native structure. Reductions were carried out in the absence of denaturant. Thus, the observed changes were not attributable to a general denaturing effect, but more specifically to cleavage of SS bonds. The products of reduction were found by gel filtration chromatography to contain both polymeric and monomeric forms. By "fingerprinting", the monomers gave no indication of having undergone SS interchange, and specific cleavage of native SS bonds was indicated by a stepwise disappearance of SS-containing peptides with increasing reduction level. Ion-exchange chromatography of the carboxymethylated monomers gave the three possible reduction intermediates, containing two, four, and six carboxymethylcysteine residues per mole, in addition to the fully reduced form. The intermediates exhibited circular dichroic (CD) characteristics that were clearly nonnative, indicating alteration of the secondary structure throughout all stages of reduction. Concomitantly, there were extensive losses of enzymatic activity at intermediate levels, while no activity was found for the fully reduced form. Partially and fully reduced lysozymes were also examined for their abilities to resume native secondary structure in the absence of SS formation, as would be evidenced by a reversion to native CD characteristics. Despite literature indications of native structure in the reduced protein as observed from examination of CD behavior, none was found in the present effort, under a variety of experimental conditions. These observations are consistent with an essential role of SS bonds in forming and maintaining a major portion of the native secondary structure in lysozyme.

There have been numerous optical studies on the protein secondary structure that remains after reductive cleavage of SS bonds, with conclusions that native secondary structure remains, to varying degrees, within the "denatured" structure. Information about this structural state could lead to a better understanding of the well-known ability of the reduced protein chain to resume the native conformation.

Yutani et al. (1968) and Ohta et al. (1971) reported from CD[1] studies that fully reduced lysozyme retained 74% and 50%, respectively, of the native α-helical content. Lee & Atassi (1973) found immunological cross-reactivity between native and fully reduced methylated lysozyme. Further, their CD and ORD studies, as well as the CD work of Tamburro et al. (1970), suggested that fully reduced lysozyme could assume a conformational state, in the absence of SS formation, that resembles the native conformation. Saxena & Wetlaufer (1970) reported that the CD behavior of fully reduced lysozyme is qualitatively similar to that of the native protein, suggesting structural relatedness. It could be concluded from these instances of similarity between structures in the native and reduced forms that the native conformation is only partially dependent on SS bonds for its existence, and this conclusion has implications for the course of events during the folding process. Thus, it appeared to Tamburro et al. (1970) that SS bonds function to stabilize the folded structure in the specific form necessary for biological activity rather than to achieve the protein conformation, which was viewed as being determined primarily by nucleation. This process involves the concept that a given amino acid sequence will form a specific secondary structure, with the ultimate conformation dictated by the tendency of the protein chain to seek the form with the lowest Gibbs free energy. This thermodynamic aspect of chain folding was developed largely through the extensive studies of Anfinsen and co-workers (1972, 1975).

More recently, there have been developments that question the thermodynamic influence as the major determinant of native conformation. Wetlaufer et al. (1974) have concluded that protein chain folding is governed by a "kinetic determinism" rather than by nucleation alone. Chavez & Scheraga (1980a,b) have, in fact, found immunological evidence for intermediate states in the refolding of ribonuclease. The studies of Creighton (1977b, 1979b) on ribonuclease, however, indicate that the transition to native conformation occurs near completion of the oxidative phase of renaturation. More specifically, Galat et al. (1981) find that closure of the third SS bond is necessary for the transition. The chain at this stage appears to undergo "simultaneous" regeneration of all measured elements of the native conformation.

These pathways differ as to the order in which SS bond formation and secondary structural transition occur. A thermodynamically governed process would permit native SS bond formation to occur as a latter event, after much of the native conformation has been determined by nucleation. A kinetically based mechanism may also achieve this effect but could integrate SS formation earlier and more essentially in the folding process. On the other hand, a simultaneous appearance of native structural characteristics would be preceded by formation of most of the SS bonds, presumably the native ones.

It remains uncertain which mode of folding would be the most generally applicable or whether the predominant pathway may differ from one protein to the next. For many SS bond containing proteins, SS formation appears to be obligatory for appearance of the completely folded and biologically active

† From the Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received May 5, 1981.*

[1] Abbreviations: Cam, carboxamidomethyl; CD, circular dichroism; Cm, carboxymethyl; CMC, carboxymethylcysteine; DEAE, diethylaminoethyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FMA, fluorescein mercuric acetate; ORD, optical rotatory dispersion; RMS, root mean square; TMA, trimethylamine; Tris, tris(hydroxymethyl)aminomethane.

protein. An understanding of the role that SS bonds play in forming and maintaining secondary structure could be helpful in establishing the relative significance of each proposed mode of folding. For the present effort, a reductive approach has been employed as a means of arriving at intermediate levels of SS content, as well as the fully reduced form, for study in relation to remaining secondary structure.

Many reported reductions have involved high concentrations of urea. This denaturant could be responsible for the pronounced alteration of secondary structure rather than SS bond cleavage per se. The lysozyme derivatives presently studied were obtained by reduction under mild conditions with use of mercaptoethanol to arrive at intermediate levels and with DTT to obtain more nearly full reduction (Bewley & Li, 1969). The partially and fully reduced forms have been studied to characterize the remaining secondary structure and to assess their abilities to assume native structure in the absence of SS bonds. Some of these results have been presented in preliminary form (White, 1980).

Materials and Methods

Full Reduction of Lysozyme and Reaction with Iodoacetate, Iodoacetamide, or Methyl p-Nitrobenzenesulfonate. For essentially full reduction in the absence of urea, 100 mg each of DTT (Bethesda Research Laboratories) and chicken egg white lysozyme (3 times crystallized, dialyzed, and lyophilized; Sigma Chemical Co.) were dissolved in 10 mL of deaerated water.[2] The solution was adjusted to pH 8.5 with aqueous TMA, and the reaction was allowed to proceed for 17 h at room temperature (22–24 °C). The precipitate was removed by centrifugation (5000*g* at room temperature) and washed 3 times with 5 mL of deaerated water. For solubilization, the protein pellet was suspended in 5 mL of deaerated water, adjusted to pH 3 with HCl, and incubated at 37 °C for 20 min. The yield of soluble protein was 45–60%. This solution could then be subjected either to gel filtration chromatography or reacted with either iodoacetate (White, 1972) or iodoacetamide as described by Tamburro et al. (1970).

Full reduction of lysozyme in 8 M urea with mercaptoethanol as the reducing agent, followed by carboxymethylation, was carried out as described earlier (White, 1972). For preparation of fully reduced lysozyme, followed by reaction with methyl *p*-nitrobenzenesulfonate to obtain the *S*-methyl derivative, the procedures of Lee & Atassi (1973) were used.

Partial Reduction of Lysozyme. Mercaptoethanol was employed under a variety of conditions to achieve reduction to intermediate levels. The protein (100 mg) was dissolved to 10 mg/mL in deaerated water, and 0.02–0.2 mL of mercaptoethanol was added (to result in concentrations of 0.03–0.3 M). The reaction mixture was adjusted with 5–25% aqueous TMA to a pH range of 7.5–8.8, and most reductions were carried out for 1.5 h, although some experiments involved 17 h of incubation. The temperature range was 25–37 °C.

Partially reduced protein precipitated from the reduction mixture during the course of reaction. This product was washed and solubilized as above for the DTT-reduced protein. As a test for completeness of removal of native lysozyme, some samples were reprecipitated and resolubilized with assays for lysozyme activity (Shugar, 1952) before and after. Reprecipitation was carried out by addition of 0.1 N NaOH, under a N_2 atmosphere, to pH 8. Then centrifugation and dissolving of the pellet were carried out as before.

[2] The deaerated water used throughout this work was prepared by passing nitrogen through water at 2–5 °C for at least 15 min prior to use.

Some experiments were conducted to investigate the protein remaining in the supernatant solution of the reduction mixture, in attempts to isolate samples at lower reduction levels. The protein was separated by acetone–HCl precipitation (White, 1972) from the supernatant solution after centrifugation (5000*g*) of the reduction mixture at room temperature. The pellet was washed 3 times with more acetone–HCl, redissolved in deaerated water, and lyophilized.

Gel Filtration Chromatography. The resin was Bio-Gel P-60 (Bio-Rad Laboratories; 100–200 mesh, with an exclusion limit of 60 000 daltons). Column dimensions were 2.4 × 80 cm, and the buffer was 0.075 M sodium phosphate–HCl, pH 3.0, described earlier (White, 1976). The column was operated at room temperature. Fraction size was 3 mL, and the flow rate was 6–10 mL/h. The protein sample (maximum 50 mg) was applied in a maximum of 3 mL, after solubilization at pH 3 as described above.

Determination of Reduction Level. So that the reduction level of the unfractionated reduced protein or of the monomeric component from gel filtration chromatography could be found, the product was reacted with iodoacetate and analyzed for CMC content, which was taken in equivalent to the SH content prior to carboxymethylation. For this reaction, a weight of iodoacetic acid equal to the weight of protein was added to the sample solution or to the pooled fractions from chromatography. The protein concentration for the former was 10 mg/mL and for the latter 0.2–0.5 mg/mL. Thus, a minimal molar ratio of 9.7 (iodoacetate:SH) was achieved (for fully reduced lysozyme). The resulting solution was made 8 M in urea (freshly recrystallized from ethanol) and adjusted to pH 8.5 under N_2 with TMA. The reaction proceeded at room temperature for 30 min. The solution was then brought to pH 3 with 1 N HCl and dialyzed against 4 L of water at 5 °C for 36 h. The membrane used was "Dialyapor" from National Scientific Co., with a molecular weight cutoff of 6000–8000. The resulting solution was lyophilized.

Hydrolysis was performed on 1–2 mg of this product in 1 mL 6 N HCl in an evacuated sealed tube for 17 h at 110 °C. The hydrolysate was dried on a lyophilizer and then subjected to analysis on a Beckman Instrument Co. amino acid analyzer, Model 120, to determine the CMC content.

Ion-Exchange Chromatography of Reduced Cm Lysozyme. The resin was DEAE-Sephadex (A-25, particle size 40–120 μm with a capacity of 3.5 ± 0.5 mequiv/g, from Pharmacia, Inc.). A slurry of this resin in water was allowed to swell overnight at room temperature. It was then adjusted to pH 9.3 with 1 N NaOH and allowed to equilibrate for 2 h with occasional further adjusting to maintain the pH. The slurry was filtered with suction and resuspended as before with a final adjustment to pH 9.3. The column (1.2 × 20 cm) was prepared with this slurry and equilibrated 2 h with 0.25 M Tris-HCl of pH 9.3. The buffer was then made 5 M with respect to urea, and this addition resulted in a dilution from 0.25 to 0.2 M with respect to Tris. It was then necessary to readjust the solution to pH 9.3 with a few drops of 1 N HCl. Column equilibration proceeded for 1 h with the urea-containing buffer. The sample (2–10 mg) of the pooled fractions from P-60 chromatography, which had been carboxymethylated (see under Determination of Reduction Level) and lyophilized, was redissolved in no greater than 1 mL of the above Tris–urea buffer and applied to the column. The flow rate was 25–30 mL/h, and the fraction size was 1.5–2.0 mL. All experiments were conducted at room temperature.

After appearance of peak I (Figure 4), gradient elution was started with the above buffer which had been modified by

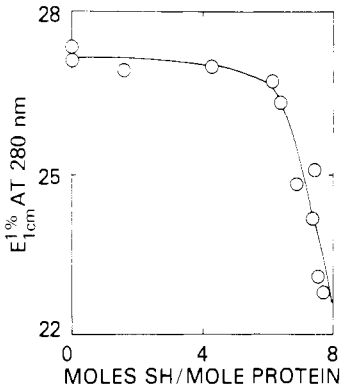

FIGURE 1: Extinction coefficients of lysozyme as functions of reduction level.

adjustment to pH 2.5 with 6 N HCl. The mixing chamber contained 250 mL, and other conditions were as described above. Elution was continued until peak IV (Figure 4) emerged.

Determination of Extinction Coefficients. These values were used for calculation of protein concentrations from optical densities at 280 nm. However, the coefficient for lysozyme is altered by reduction, and therefore it was necessary to study the value as a function of reduction level.

For the reduced products prior to reaction with iodoacetate, the protein concentration was found by the method of Lowry et al. (1951) and related to optical density at 280 nm to obtain values for $E^{1\%}_{1cm}$. Both native and reduced Cm lysozymes were assayed as a check on the method, with extinction coefficients of 27.1 (Ehrenpreis & Warner, 1956) and 22.7 (White, 1976), respectively. This method of assay yielded results for the two proteins agreeing within ±5%. The reduction levels were found by CMC analysis as described above. From a series of experiments the relationship in Figure 1 was developed, and from the resulting smooth curve the coefficients were read for all samples prepared subsequently. In preliminary experiments, this relationship did not vary significantly in comparison of unfractionated samples with monomeric components isolated by gel filtration, at reduction levels of approximately 4, 6, and 8 CMC/mole. Therefore the relationship of Figure 1 was assumed to apply with equal validity to the fractionated samples for the purpose of calculating column yields from gel filtration studies.

For Cm derivatives of partially reduced lysozyme that were isolated by DEAE-Sephadex chromatography, the extinction coefficients were determined by relating optical density at 280 nm to the concentration. The latter value was calculated from the total recovery of amino acid content on amino acid analysis of an appropriate aliquot, which was first hydrolyzed in 6 N HCl as described under Determination of Reduction Level.

Proteolytic Digestion of Lysozyme and Reduced Derivatives and Two-Dimensional Separation of Peptides ("Fingerprinting"). Lysozyme, after reduction to various levels and carboxymethylation, was digested with nagarse (Nagase and Co., Osaka, Japan, obtained through Enzyme Development Corp., NY; batch no. 7735069, crystalline). Thus, 10 mg of the lyophilized sample was suspended in 1 mL of 0.1 M ammonium bicarbonate, and 0.1 mL of the nagarse solution (1 mg/mL in water) was added. Digestion proceeded 6 h at 37 °C, and the digest was lyophilized.

For fingerprinting, the lyophilized digest was dissolved in water to 20 mg/mL, and 0.5 mL was dried on a spot of approximately 0.5 cm in diameter on Whatman 3MM paper which was then chromatographed ascending in the organic layer of butanol–glacial acetic acid–water (4:1:5). After drying at room temperature, the paper was subjected to electrophoresis at a gradient of 70 V/cm in pyridine–acetate buffer of pH 3.5 (Katz et al., 1959). After drying at room temperature, the peptides were located by dipping in a 1% solution of ninhydrin in absolute ethanol, followed by drying at 80 °C in a ventilated oven. The SS-containing spots were detected on a duplicate paper by the fluorescence-quenching method of Karush et al. (1964).

Random Reoxidation of Lysozyme in Urea. Fully reduced lysozyme, prepared as described above, was dissolved to 10 mg/mL in 8 M urea and allowed to reoxidize by exposure to air in an open beaker with stirring at room temperature for 5 h at pH 8.5, adjusted with TMA. The product was precipitated with acetone–HCl as described earlier (White, 1972), centrifuged at 12000*g* at 5 °C, washed 2 times with this solvent, redissolved in water, and lyophilized. Completeness of reoxidation was confirmed by titration with DTNB (Habeeb, 1972), by which no remaining SH groups could be detected.

Preparation of Solutions of Reduced Lysozyme for CD Study. For study prior to blocking of the SH groups, the reduced samples were chromatographed as described under Gel Filtration Chromatography. The peak fraction of the monomer was diluted to 0.3–0.4 mg/mL with 0.075 M phosphate–HCl buffer of pH 3 (White, 1976) and centrifuged at 12000*g* for 20 min at 5 °C. These dilutions were in the range 10–15-fold. This solution was used for CD study.

For investigation of CD behavior at higher pH values, dilution was made in water, instead of buffer, and the final protein concentration was 0.03–0.04 mg/mL. Adjustment to pH 7–8 was made, after passage of N_2 through the sample for 15 min at room temperature, by addition of 0.03 mL of 0.1 M ammonium bicarbonate to 5 mL of sample solution. The solution was placed in a quartz cuvette of 10-mm path length, and examination by CD was begun immediately. It was noted that attempts to raise the pH of a solution in the phosphate–HCl buffer used above resulted in precipitation of the protein. So that the possibility of oxidation at the higher pH level could be checked, aliquots before and after pH adjustment, as well as after CD study, were titrated with DTNB (Habeeb, 1972). No significant differences were found; hence, there was no indication that oxidation might have been a complicating factor in these studies.

Preparation of the Cm, Cam, or *S*-methyl derivatives for CD study involved redissolving each sample from the lyophilized state in water to approximately 20 mg/mL, followed by dilution, as above, with phosphate–HCl buffer.

Determination of protein concentration for the partially reduced samples was made from the relationship between extinction coefficient and reduction level (Figure 1). For the native protein, a value of 27.1 for $E^{1\%}_{1cm}$ was used (Ehrenpreis & Warner, 1956). For the fully reduced protein, whether produced as the Cm, Cam, or *S*-methyl derivative, the value was assumed as 22.7 (White, 1976).

For CD study after ion-exchange chromatography, the fractions of each peak from chromatography of reduced Cm samples on DEAE-Sephadex were pooled and subjected to dialysis against 4 L of water at 5 °C for 17 h and then with a fresh change of water for 6 h. Each sample was lyophilized. For solubilization, the sample was suspended in 5 mL of water, and 0.1 N HCl was added to a pH of 2.5–3.0. Dissolving was effected on incubation at 37 °C for 15–30 min. The sample was centrifuged for 30 min at 12000*g* and then appropriately diluted with 0.075 M phosphate–HCl buffer for activity assay and CD studies.

CD Studies. The instrument employed was a Cary 60 recording spectropolarimeter with a Model 6001 CD attachment. Studies were carried out at 2 and 27 °C in quartz cuvettes of 1- or 10-mm path length.

Computer curve fitting of the CD data, to obtain estimates of structural fractions, was carried out as described earlier (White, 1976). Ellipiticity values were calculated for 14 points, from 207.5 to 240 nm, at intervals of 2.5 nm. An average of three CD curves was taken for each sample. The polylysine basis spectra were those of Greenfield & Fasman (1969). Alternatively, the basis spectra of Chen et al. (1974) were used, with the average chain length of α helix taken as 11 residues. The goodness of fit of the CD data with the computer-reconstructed ("best-fit") curve was represented by the RMS error, discussed earlier (White, 1976) and given as degree centimeter squared per decimole.

The mean residue weights for calculation of ellipticities were 111.0 and 114.6 for native lysozyme and its fully reduced, derivatized forms (Cm and Cam), respectively. For samples at intermediate levels of reduction, studied by CD prior to carboxymethylation, the value for native lysozyme was used. For intermediate levels after carboxymethylation, the values used were intermediate between that of the native protein and that of its fully reduced Cm form, corresponding to the reduction level as measured by the CMC content.

Lysozyme Activity Assay. Assays at the various reduction levels studied were conducted as described by Shugar (1952), except that 0.1 M phosphate–HCl buffer of pH 4 was used. It had been determined that this buffer permitted all samples to remain soluble and circumvented the possible reoxidizing effects that would have been a complicating factor at higher pH levels under which the assay is more usually performed.

Samples assayed prior to carboxymethylation were taken from the peak fractions of the monomers from gel filtration chromatography (Figure 2) by diluting appropriately into the pH 4 buffer. An aliquot of the diluted solution was taken directly for assay. Activities were related to that of native lysozyme, assayed simultaneously, to obtain the percent of specific activity of the native enzyme.

Results

A variety of conditions were explored for the reduction of lysozyme with the object of obtaining products over a broad range of reduction level that could be studied in relation to effects on secondary structure. The common feature among the following conditions was the absence of denaturant.

Effects of pH. There was a marked influence of pH on the yield and extent of reduction. The maximum yield, seen at pH 8.5 (Table I, experiments 3 and 4), coincided with the highest levels of reduction achieved with mercaptoethanol. Lower yields and possibly lower reduction levels are seen at pH 8.3–8.4 (experiments 1 and 2). There was a similar reduction level but lower yield at pH 8.8 (experiment 5). At pH 7.5 (not shown) there was no significant yield of reduced protein.

Effects of Reducing Agents. Reduction with mercaptoethanol resulted in levels between 4.4 and 6.7 SH/mol (Table I). A concentration of 0.14 M for this reagent was used for most experiments, and there was no greater yield or higher level of reduction at 0.3 M (experiment 11). At 0.03–0.06 M, yields and levels of reduction were clearly lower (experiments 9 and 10). The use of DTT, in agreement with Bewley & Li (1969), gave nearly full reduction of lysozyme in the absence of denaturant (experiments 13–15).

Effects of Time and Temperature. At pH 8.5 the yield of reduced protein and the level of reduction were considerably greater at 37 °C (experiments 3 and 4) than at either 32 (experiment 7) or 45 °C (experiment 8), while the levels of reduction were similar. Increasing the reaction time to 17 h at a lower temperature (25 °C; experiment 6) resulted in lower yields and reduction levels. A higher temperature (37 °C) for the same time interval (not shown) did not significantly increase the yield or reduction level.

A lower temperature (32 °C; experiment 12), with 0.06 M mercaptoethanol, produced close to 4 SH/mol. These conditions, at pH 8.5, were used to produce further samples for study near this level of reduction (see Ion-Exchange Chromatography of Reduced Cm Lysozyme).

Gel Filtration Chromatography. Chromatography of partially reduced lysozyme usually yielded three components (Figure 2). There was no obvious correlation between the level of reduction and the relative sizes of the peaks. Peak II was always the smallest and, on occasion, was absent. Peaks I and III were most often present in approximately equal amounts.

It is curious that peak III (Figure 2) eluted identically with native lysozyme. This finding was verified by mixing un-

Table I: Investigation of Conditions for Reduction of Lysozyme

expt	pH	concn of reducing agent (M)[a]	temp (°C)	yield[b] (%)	CMC/mol[b,c]
1	8.3	0.14	37	12	5.2
2	8.4	0.14	37	18	6.4
3	8.5	0.14	37	42	5.8
4	8.5	0.14	37	49	6.7
5	8.8	0.14	37	24	6.1
6[d]	8.5	0.14	25	33	4.8
7	8.5	0.14	32	18	4.6
8	8.5	0.14	45	18	5.8
9	8.5	0.03	37	7	4.5
10	8.5	0.06	37	11	5.0
11	8.5	0.30	37	42	5.9
12	8.5	0.06	32	12	4.4
13	8.4	0.06	27	50	7.8
14	8.5	0.06	27	47	7.6
15	8.5	0.06	27	53	7.7

[a] The reducing agent for experiments 1–12 was mercaptoethanol; that for experiments 13–15 was DTT. [b] Yields of solubilized precipitate (see Materials and Methods) were determined from optical density at 280 nm with extinction coefficients as given in Figure 1. The protein remaining undissolved and that remaining in the supernatant solution of the reduction mixture are excluded from the yield. The yield was calculated as the percent of the initial protein in the reduction mixture. [c] The SH content was assumed equivalent to CMC content which was found, after carboxymethylation, by amino acid analysis as described under Materials and Methods. [d] Incubation time of the reduction mixture was 17 h. The time for all other experiments was 1.5 h.

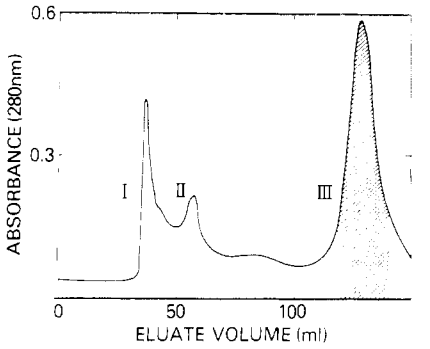

FIGURE 2: Chromatography of partially reduced lysozyme (5.4 SH/mol) on Bio-Gel P-60. The cross-hatched area indicates the pooling of fractions for subsequent study of the monomer. Column yields were 80–90%.

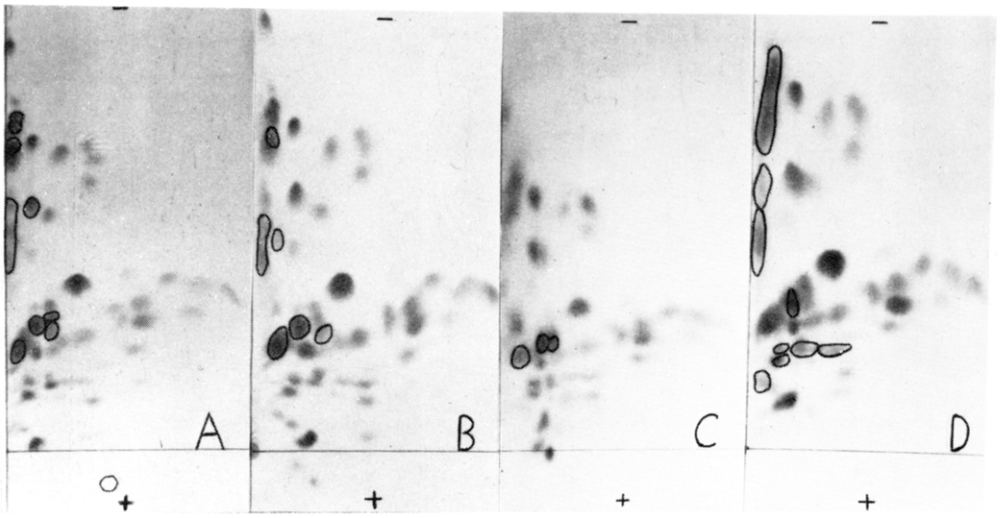

FIGURE 3: Two-dimensional separation of peptides from proteolytic digests of (A) native lysozyme, (B) carboxymethylated peak III (Figure 2) of partially reduced lysozyme (4.4 CMC/mol), (C) carboxymethylated peak III of partially reduced lysozyme (5.6 CMC/mol), and (D) urea-reoxidized lysozyme. Origin is at lower left of each paper. First dimension (chromatography) is shown horizontally and second dimension (electrophoresis) vertically. FMA-positive spots (with SS-containing peptides) are encircled. These papers were developed with ninhydrin.

Table II: Yields, Reduction Levels, and Extinction Coefficients of Components Separated by Ion-Exchange Exchange Chromatography[a] of Reduced Cm Lysozyme

col-umn	I[b]			II			III			IV		
	yield[c] (%)	CMC[d]	E^e	yield (%)	CMC	E	yield (%)	CMC	E	yield (%)	CMC	E
A	23.0	1.8	28.5	23	4.4	26.4	26	6.1	26.9	28	7.8	23.1
B	2.0	2.1	27.1	23	3.8	26.8	57	6.2	26.4	18	8.0	22.7
C							15	6.3	26.5	85	7.9	22.3

[a] All data apply to the separated components of Figure 4. [b] Roman numerals refer to peak numbers of Figure 4. [c] Yields are expressed as percent of total weight of eluted peaks. For this determination, the weight of each peak was found from the concentration of pooled fractions, with the appropriate extinction coefficient used as shown in the table. [d] Reduction level of each peak was found as described in text and is expressed as moles of CMC per mole of protein. [e] Extinction coefficients ($E^{1\%}_{1cm}$) were determined as described in text.

fractionated partially reduced lysozyme with an equal weight of native protein. The resulting peak III retained the same shape, with no indication of an additional component. Thus, the reduced protein in peak III did not differ sufficiently in Stokes' radius from that of the native protein to allow detection by this means of chromatography. Similar experiments have been conducted with DTT-reduced lysozyme and with fully reduced Cm lysozyme. No deviation from native behavior was seen for the major component in any of these samples on Bio-Gel P-60.

Experiments were conducted with substitution of Craig's buffer (Acharya & Taniuchi, 1978) of pH 4.8 for the above phosphate buffer. The results (not shown) agreed with those of Acharya & Taniuchi (1978) insofar as the reduced lysozyme samples were eluted at a volume lower than that for native lysozyme. The samples employed were DTT-reduced lysozyme and partially reduced Cm lysozyme at 5.6 CMC/mol. Therefore the results suggest higher hydrodynamic volumes for the fully and partially reduced forms when chromatographed with the buffer used by Acharya & Taniuchi (1978) and are consistent with their results for the fully reduced form.

Fingerprinting Studies. Various reduced, carboxymethylated samples were subjected to proteolytic digestion and two-dimensional separation of peptides. There were two objectives. The first was to search for extensive SS interchange, which could have been a complicating factor in studies of secondary structure. The second was to search for indications of specific SS bond cleavage, which would be shown by increasingly smaller numbers of SS-containing spots at higher reduction levels, whereas a sameness of SS patterns throughout all reduction levels would show random cleavage.

Typical fingerprinting results are given in Figure 3. No SS interchange is indicated in comparison of the partially reduced samples B and C with the randomly urea-reoxidized sample D. For the latter, some of the FMA-positive spots appear as smears rather than discrete spots, and these coincide with smears of ninhydrin-positive material which may contain many of the peptides possible from random combination of half-cystine residues. Among the partially reduced samples, of which samples B and C are representative, there was no indication of the multiplicity of FMA-positive material characteristic of random pairing. The positions of the SS peptides of the partially reduced samples did not differ from those of the native sample (A).

Specificity of SS bond cleavage is indicated by the decreasing number of SS-containing spots from sample A to sample C. The pattern in sample C is characteristic of reduced lysozyme between 5.6 and 6.7 CMC/mol, with no obvious differences in this range.

In contrast, fingerprinting of peaks I and II of Figure 2 (not shown) revealed numerous additional FMA-positive spots, which coincided with ninhydrin-positive material. The presence of these components may indicate extensive intermolecular SS interchange, which would account for the apparent polymerization suggested by their lower elution volumes.

In summary, fingerprinting studies indicate specificity of SS bond cleavage, the details of which remain to be explored, while there is no evidence for random SS interchange within the monomer samples.

Ion-Exchange Chromatography of Reduced Cm Lysozyme. The samples generated as described here are resolvable into the four possible components, at the one-fourth-reduced,

Table III: Curve Fitting of Lysozyme at Various Levels of Reduction

reduction level (CMC/mol)	expt[a]	structural fractions[b]							
		polylysine data				Chen et al. (1974) data			
		α	β	RMS[c]	α/β[d]	α	β	RMS	α/β
0.0	native	31[e]	11	835	1.0	33	12	720	1.0
4.4	12	9	32	592	0.10	121	13	495	0.34
4.5	9	6	30	637	0.011	13	16	666	0.30
4.6	7	8	42	475	0.067	23	21	1083	0.40
4.8	6	14	39	620	0.13	25	24	718	0.38
5.0	10	11	27	647	0.14	17	22	596	0.28
5.2	1	17	32	270	0.19	28	25	464	0.41
5.8	3	12	28	389	0.15	19	18	373	0.39
5.8	8	10	36	331	0.10	23	24	684	0.35
5.9	11	6	40	305	0.053	20	22	781	0.33
6.1	5	12	30	389	0.14	18	15	624	0.44
6.4	2	3	37	343	0.029	18	20	706	0.33
6.7	4	6	34	422	0.062	18	22	651	0.30
7.6	14	6	35	494	0.061	17	20	812	0.31
7.7	15	61	41	311	0.052	20	21	789	0.35
7.8	13	3	40	409	0.027	12	15	992	0.29

[a] Experiment numbers are those in Table I but are presented in the order of increasing reduction level. [b] Structural fractions were obtained by curve fitting as described in the text with basis spectra obtained from polylysine (Greenfield & Fasman, 1969) or from Chen et al. (1974). [c] Goodness of fit between experimental and computer-reconstructed curves is expressed as degree centimeter squared per decimole. [d] Ratios of percent chain length as α helix to percent chain length as β structure were normalized. Thus, the values obtained with structural fractions from the polylysine and Chen et al. data were divided by the respective ratios for native lysozyme. [e] The ellipticities used for determination of structural fractions were averages from three experimental CD curves which were obtained identically. When individual curves were so processed, the resulting fractions differed within ±3% of the chain length.

half-reduced, three-fourths-reduced, and fully reduced levels (Figure 4 and Table II).

The distribution of components is nearly equal at the lowest level (Figure 4A), whereas peak III is heavily favored at the higher level in Figure 4B. The half-reduced component (peak II), on the other hand, changed relatively little. The DTT-reduced protein (Figure 4C) shows predominantly the fully reduced form (peak IV) with relatively little of the three-fourths-reduced product and nothing else.

Extinction coefficients of the isolated components (Table II) confirm the relationship in Figure 1, since they indicate that an approximately native value is maintained until the three-fourths level is reduced.

In separate experiments, protein isolated from the supernatant solution of the reduction mixture (prepared as for experiment 12, Table I) by acetone–HCl precipitation and then subjected to carboxymethylation (to give 1.8 CMC/mol) was chromatographed on DEAE-Sephadex. Only peak I emerged, with traces of material at the approximate positions for peaks II–IV. Amino acid analysis of peak I indicated that the sample was devoid of CMC content. Hence, the predominant component of the supernatant solution was native lysozyme, with no indication of partially reduced derivatives that might have been soluble under the reduction conditions employed.

In conclusion, all levels of reduction have been demonstrated to exist in the samples produced by the presently described reduction methods.

CD Studies. The monomers from gel filtration chromatography have been studied by CD before and after exposure to the denaturing conditions necessary for carboxymethylation and ion-exchange chromatography. This effort was undertaken to see whether native structure might be left intact after cleavage of SS bonds under the benign conditions employed.

From the representative spectra in Figure 5, it is clear that the partially reduced samples (prior to carboxymethylation) differ markedly from the native protein, appearing more similar to the fully reduced protein. Table III summarizes structural fractions found from curve fitting of CD data. The fractions for native lysozyme are in good agreement with those from X-ray diffraction studies. The goodness of fit between

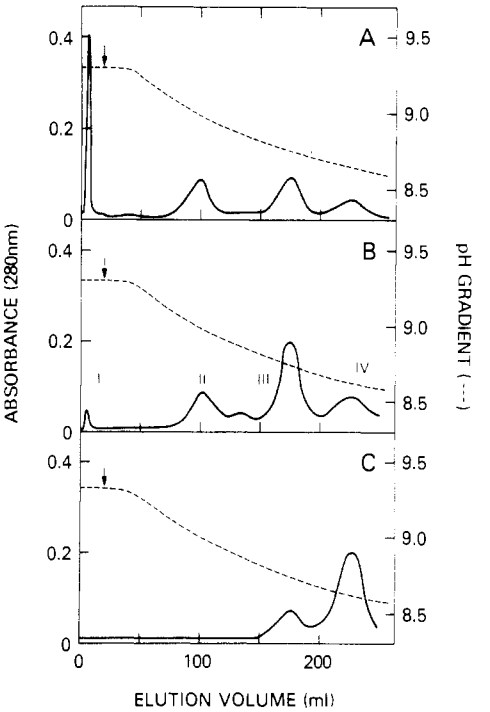

FIGURE 4: Chromatography of reduced Cm lysozyme on DEAE-Sephadex. The reduction levels prior to chromatography were 4.4, 5.8, and 7.6 CMC/mol of protein for (A), (B), and (C), respectively. Numbering of peaks for sample B with Roman numerals applies to all three samples. Column yields varied between 70% and 90%. Arrows denote start of gradient elution.

computer and experimental curves, as expressed by the RMS error, is slightly better with the Chen et al. (1974) data than with the polylysine (Greenfield & Fasman, 1969) data. For the reduced samples, however, the RMS errors with the polylysine set are consistently lower (270–647), indicating improved agreement between experimental and computer-reconstructed curves. With the Chen et al. data, the range is 373–1083, and hence there is no consistently improved fit.

The structural changes occurring with reduction are conveniently summarized by the normalized ratio of percent chain

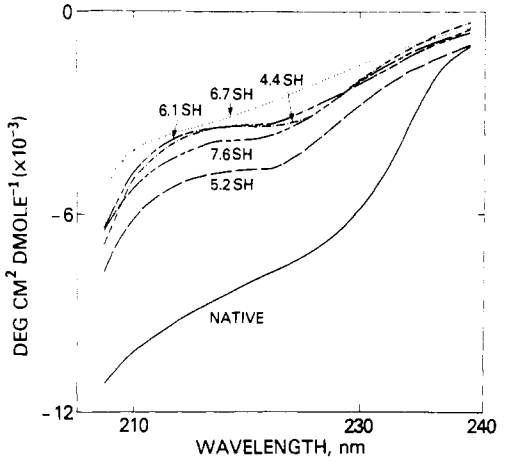

FIGURE 5: Typical CD curves for native and reduced lysozymes. All samples were studied in 0.075 M sodium phosphate–HCl buffer of pH 3. For the SH levels of 4.4, 5.2, 6.1, 6.7, and 7.6, the selected samples were from experiments 12, 1, 5, 4, and 14, respectively, as given in Table I.

Table IV: Structural Fractions of Peaks from DEAE-Sephadex Chromatography

peak[b]	structural fractions[a]		
	α helix	β structure	RMS[c]
IA	−2	34	320
IIB	−1	34	312
IIIB	0	37	529
IVB	0	35	318

[a] Structural fractions were obtained by curve fitting with the polylysine data of Greenfield & Fasman (1969) and are expressed as percent of chain length. [b] Roman numerals and letters refer to the peak number and sample, respectively, resulting from ion-exchange chromatography (Figure 4). Peaks I, II, III, and IV represent the one-fourth-, half-, three-fourths-, and fully reduced products, respectively. [c] Goodness of fit between experimental and computer-reconstructed curves is expressed as the root mean square error in degree centimeter squared per decimole.

length as α helix to that as β structure (Table III). This value drops with partial reduction to a range of 0.28–0.44 when the Chen et al. (1974) data are used and 0.03–0.19 for the polylysine data. The higher values found with the former reflect both the higher helix and lower β structure values obtained with that set as shown in Table III. These ratios, as well as the corresponding structural fractions, indicate that a marked structural change has occurred, even at the lowest reduction level, with little or no further change as reduction approaches completion.

The considerable variability of the structural fractions among the reduced samples in Table III, which also is reflected in the ranges of α/β values, is attributable to variations from one sample to the next, for reasons remaining to be explored, rather than to experimental error. The reproducibility of CMC values from amino acid analysis and of structural fractions from curve fitting is excellent. For the former, an error of ±3% is usual. The fractions from repeated CD examination are reproducible within ±3% of the chain length.

The CD analyses on the separated peaks (Table IV) show uniformity in structural fractions from the lowest to the highest CMC content in comparison to the unfractionated samples of Table I. Moreover, the helical content is nearly nonexistent, and thus it appears that additional structural changes may have resulted, either from exposure to urea, from introduction of negative Cm groups, or from both. Although the content of β structure appears less variable, it is still similar to the values of Table I and continues to show consistent increases over the

Table V: Enzymatic Activities of Reduced Lysozyme[a]

sample	activity (%)[b]
9[c]	6 ± 2
7[c]	5 ± 2
11[c]	2 ± 1
13[c]	0
IA[d]	14 ± 4
IIB[d]	8 ± 2
IIIB[d]	6 ± 2
IVB[d]	0

[a] Enzymatic activity assays were performed as described under Materials and Methods. [b] Activities are expressed as percent of the specific activity of native lysozyme. All assays were performed in duplicate. [c] Sample numbers correspond to the experiment numbers of Table I. These assays were performed on the SH form. Reduction levels were subsequently determined by carboxymethylation and CMC analysis, as described under Materials and Methods. [d] These assays were performed on peaks I–IV resulting from ion-exchange chromatography (Figure 4). Roman numerals and letters refer to peak numbers and samples, respectively.

native value. Therefore, although some further changes have occurred, they appear relatively minor compared to those from partial reduction prior to carboxymethylation.[3]

Curve fitting of the separated components with the data of Chen et al. (1974) (not shown) resulted in RMS errors of 1600 to over 2000 deg cm^2 dmol^{-1}, an indication that this set is inappropriate for the samples of Table IV.[4] By contrast, the RMS errors obtained with the polylysine set (Table IV) are the lowest seen in this work.

Enzymatic Activities of Reduced Lysozyme Samples. As an additional measure of conformational integrity, samples from experiments in Table I were selected for lysozyme activity assay (Table V). The resulting small activities decreased with increasing reduction level, while no activity could be detected for the fully reduced form. Thus, the conformational structure that forms the active site is mostly, but not entirely, destroyed by partial reduction, while this structure is completely eliminated after reduction with DTT. The components of these samples, separated by ion-exchange chromatography after carboxymethylation, also show this trend (Table V).

Attempts To Achieve Native Structure in the Absence of SS Bonds. The experiments thus far described were conducted at pH 3–4. In further experiments, the effects of higher pH levels were studied, since it is from slightly alkaline solution that reduced lysozyme regenerates to the native form. The CD behavior for fully reduced lysozyme at pH 7–8 is similar to that of the partially reduced form (Table IV). Such curves were difficult to reproduce, possibly because of the lack of pH control at the low salt concentrations employed. Fitting of the CD curves at pH 7–8 gave less reliable results (Table VI), as evidenced by the higher RMS errors with both sets of basis spectra in comparison with those from samples at lower pH values. The trend, however, toward lower helix and higher β structure was still in evidence. More significantly, there was

[3] That Cm groups may cause additional structural change was indicated earlier by the differences in CD properties of reduced lysozyme, dependent upon whether a negative, positive, or neutral group was substituted onto the sulfur (White, 1976), as well as by the recent work of Chavez & Scheraga (1980b), whereby "K_{conf}" of reduced ribonuclease changes further on carboxymethylation.

[4] In the experience of this laboratory (unpublished results), RMS errors of the magnitude observed in these experiments for a number of native proteins have been seldom accompanied by meaningful agreement between structural fractions elaborated by CD study and those found by X-ray diffraction.

Table VI: Structural Fractions of Reduced Lysozyme under Various Conditions of Temperature, pH, and Solvent

conditions and samples	structural fractions[a]					
	polylysine data[b]			Chen et al. (1974) data		
	α	β	RMS[c]	α	β	RMS
pH effects[d]						
pH 3, fully reduced[e,f]	9, 10	36, 35	444, 471	23, 24	23, 23	931, 1106
pH 7-8, fully reduced[f,g,h]	5, 6	42, 43	1250, 977	20, 20	22, 23	1094, 958
pH 3, partially reduced[e,i]	9, 10	23, 29	501, 492	16, 12	4, 10	416, 527
pH 7-8, partially reduced[g,h]	6, 6	42, 40	1322, 950	11, 13	15, 18	1579, 162
pH 3, native	30, 31	11, 13	811, 749	37, 36	12, 14	683, 701
pH 7-8, native[f,g,h]	20, 22	26, 26	328, 345	29, 32	16, 15	433, 486
temperature effects						
2 °C, reduced Cam lysozyme[j,k]	10, 13	26, 23	476, 505	14, 16	20, 17	460, 410
27 °C, reduced Cam lysozyme[j,k]	11, 10	28, 29	375, 412	17, 16	14, 14	656, 521
2 °C, fully reduced[e,f,l]	8, 10	34, 37	521, 473	23, 25	22, 25	940, 899
2 °C, partially reduced[e,f,l]	9, 9	25, 34	419, 523	12, 12	18, 15	426, 415
methanol effects[d]						
33% methanol, with reduced Cam lysozyme[j,k]	13, 15	50, 53	438, 577	42, 38	43, 44	1294, 1128
0% methanol, with reduced *S*-methyl lysozyme[j,m]	9, 10	17, 16	420, 478	39, 37	45, 56	1450, 1391
35% methanol, with reduced *S*-methyl lysozyme[j,n]	10, 10	49, 48	548, 691	24, 21	26, 27	1409, 1221
0% methanol, with native lysozyme[j,n]	32, 30	11, 12	606, 650	37, 40	9, 12	651, 712
35% methanol, with native lysozyme[j,n]	31, 30	12, 12	611, 680	36, 37	11, 12	670, 629

[a] Structural fractions are expressed as percent of the chain length. [b] Curve fitting to obtain structural fractions was done with the basis spectra from polylysine as given by Greenfield & Fasman (1969) and with the calculated spectra of Chen et al. (1974). [c] Goodness of fit between experimental and computer-reconstructed CD curves is expressed as degree centimeter squared per decimole. [d] Studies were carried out at 27 °C. [e] Samples were in 0.075 M sodium phosphate–HCl buffer of pH 3. [f] Lysozyme was reduced with DTT as described in the text. [g] Each pair of fractions represents experiments on two separately reduced samples. [h] Solutions were adjusted to the pH 7-8 range with NH_4HCO_3 as described in text. [i] Partial reductions were carried out as for experiments 5 and 6, Table I, followed by carboxymethylation as described in text. [j] Each pair of fractions represents experiments on two separately prepared solutions of the same sample. [k] The conditions for preparation and study of this derivative of fully reduced lysozyme were those of Tamburro et al. (1970). [l] These experiments were conducted in 0.075 M sodium phosphate–HCl buffer of pH 3. For comparison with the corresponding experiments at room temperature, see under "pH effects", this table, for the pH 3, partially and fully reduced samples. [m] The conditions for preparation and study of this derivative of fully reduced lysozyme were those of Lee & Atassi (1973). [n] The conditions of CD study of native lysozyme are essentially those of both Tamburro et al. (1970) and Lee & Atassi (1973), and these experiments therefore serve as controls for both the Cam and *S*-methyl derivatives.

no indication of a return to native CD behavior as a result of raising the pH under the nonoxidizing conditions employed. For this comparison it is necessary to examine the structural fractions for native lysozyme (Table VI) within the higher pH range, and it can be seen that, although there is some change from raising the pH, there is no similarity that might suggest a return of the reduced samples to the structural form that obtains for the native protein in this pH range.

Tamburro et al. (1970) modified CD behavior of fully reduced Cam lysozyme in water solution by lowering the temperature to 2 °C and also by adding methanol, and these changes suggested a return to native conformation. In the present effort, however, no change could be seen with temperature for the Cam derivative (Figure 6 and Table VI), for the *S*-methyl derivative of Lee & Atassi (1973) (Table VI), or for the DTT-reduced and partially reduced samples (Table VI).

The effects of methanol included a marked increase in amplitude, with a minimum of −13 300 deg cm^2 dmol^{-1} at 217 nm for DTT-reduced lysozyme and a smaller one of −9000 deg cm^2 dmol^{-1} for reduced Cam lysozyme in water solution [conditions of Tamburro et al. (1970)], also at 217 nm (Figure 6). The effects of methanol on CD behavior of the *S*-methyl derivative under the conditions of Lee & Atassi (1973) (curve not shown) were similar to those on the Cam derivative under the conditions of Tamburro et al. (1970) (Figure 6).

The CD curve of the latter derivative with 33% methanol (Figure 6) resembled that given by Tamburro et al. (1970). Curve fitting (Table VI) produced small increases in helix and larger increases in β sheet. For example, compare "27 °C reduced Cam lysozyme" and "33% methanol with reduced Cam lysozyme". As a control, native lysozyme in 35% methanol showed no significant difference in its CD behavior

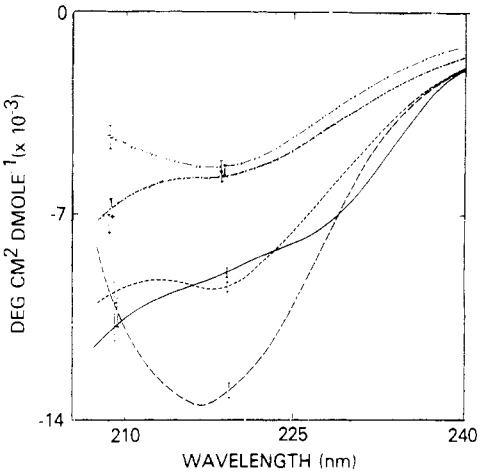

FIGURE 6: CD of lysozyme and its derivatives under various conditions examined for their potential in developing native structure. (—) Native lysozyme; (−−) DTT-reduced lysozyme in 33% methanol; (−·−) Cam lysozyme in 33% methanol; (···) Cam lysozyme in H_2O, 2 °C; (···) Cam lysozyme in H_2O, 27 °C; (−···) DTT-reduced lysozyme in H_2O, 27 °C.

from that in aqueous solution (Table VI). Thus, there is no verification for the finding of Tamburro et al. (1970) that the Cam derivative returns to the native structure in methanol.

Other variations (not shown) tried for the DTT- and mercaptoethanol-reduced samples were 33% methanol at room temperature and 2 °C and also pH 7-8 at 2 °C. However, no CD changes were observed that were any more indicative of a return to native structure than those shown in Table VI and Figure 6.

In conclusion, there is little, either from visual examination of these spectra or from curve fitting, that would suggest

similarity to native structure. A major return to the native state in the absence of SS bond formation, therefore, could not be substantiated under the conditions examined.

Discussion

Evaluation of CD as a Means of Studying Secondary Structure Changes during Denaturation. Visual examination of the CD spectra presented here shows resemblance between fully reduced lysozyme and the partially reduced forms, with no apparent tendency to revert to native behavior. The significance of the present results rests largely on these observations. Therefore, it was deemed appropriate to examine the data more closely with curve fitting, by which estimates might be made of the structural fractions at various levels of reduction. However, the application of this method to CD data is not without theoretical and practical difficulties, some of which were discussed previously (White, 1976).

In the case of lysozyme, the structural fractions, as elaborated by X-ray diffraction studies (Blake et al., 1967), are closely approximated by curve fitting with either set of basis spectra employed. The changes that occur in CD behavior on reduction of lysozyme are, therefore, the subject of concern, and it must be questioned whether the applicability of CD to the products is as meaningful as for the native protein.

Contributions from the aromatic residues tyrosine, phenylalanine, and tryptophan to the CD spectrum of native lysozyme do not appear significant within the range of wavelength studies. Their ellipticities coincide at 222 nm, and the resulting contribution could be taken as a first approximation, according to Sears & Beychok (1973), for evaluating secondary structure in this region. For lysozyme, on a residue basis, this contribution would approximate +1200 deg cm^2 dmol^{-1}. With an observed ellipticity for native lysozyme of −9010 deg cm^2 dmol^{-1} at 222 nm, this value could be significant. The negative contribution below this wavelength from tryptophan may account for the insignificance of the aromatic contribution to the native spectrum. With the diminished ellipticity of reduced lysozyme, on the other hand (typically −5500 deg cm^2 dmol^{-1} at 222 nm), the aromatic contribution would be about 22% of the total. Thus, it is likely that aromatic contributions could significantly distort the spectrum of reduced lysozyme. For a precise treatment of the reduced protein CD spectrum, it would be necessary to take into account the effects of secondary structural alteration on μ–μ coupling interactions that are associated with the aromatic residues. At the present level of development of this subject, a quantitative treatment would not be feasible.

Much study has been devoted to the rotatory effects of SS bonds, which depend largely on the "screw sense" associated with the dihedral angles about the sulfurs (Sears & Beychok, 1973). The contributions of SS bonds to CD in the far-UV, at least for some proteins, do not appear to complicate those from secondary structure (Bewley, 1977; Leach et al., 1974), nor do they affect the CD behavior of native lysozyme. For the latter protein, two of the SS bonds are skewed oppositely from the remaining two, and thus their rotatory effects cancel (Blake et al., 1967). Not so easily dismissed are the possible contributions from SS bonds in the partially reduced protein. It is conceivable that reduction could result in cleavage of specifically skewed bonds, leaving a rotatory contribution from the remainder. However, the resemblance in CD spectra between partially and fully reduced lysozymes (Table IV) does not suggest a significant distortion in the partially reduced samples from this source.

Polymerization, suggested by Sawyer et al. (1971) as an explanation for an increase in the β structure seen by ORD and CD after thermal denaturation of bovine lactoglobulin, did not contribute to the presently observed rise in β structure, since the samples studied were monomers.

Another potential problem is SS interchange, with partial reduction, that might result in rearrangement of secondary structure. However, there is no indication of SS interchange from "fingerprinting" of the partially reduced monomers.

The relative merits of the basis spectra sets employed must also be considered. The Chen et al. (1974) spectra have found wide use in curve fitting. However, the fact that these data are derived from native proteins may suggest poor suitability for the present study, since it is well recognized that the unordered moiety of a native protein cannot be equated with the randomly coiled structure of a fully denatured protein (Jirgensons, 1973). There is sufficient evidence, however, of nonrandom behavior in reduced lysozyme (Yutani et al., 1968; Lee & Atassi, 1973; White, 1976) to give indication that this product may retain elements of ordered structure and therefore that its CD behavior could be compatible with the Chen et al. data.

Polylysine is historically significant as an early source of basis spectra. The data obtained from this polymer, however, are not currently in wide use, since the CD behavior of a polyamino acid may differ in many respects from that of a protein. It is worth noting, however, that the fits are consistently improved between the experimental and computer-reconstructed CD curves (shown by decreasing RMS errors) for reduced lysozyme samples in comparison to the native protein. The improvement could be for one or both of two reasons: (1) removal of SS bonds could produce an aperiodic moiety in the reduced chain more similar to that of polylysine, in the respect that neither is affected by tertiary constraints; (2) various interfering coupling interactions (Strickland, 1974) that contribute to native CD behavior could be altered or removed as the relatively rigid native conformation is relaxed by elimination of SS bonds.

By comparison, the Chen et al. (1974) data show no consistently improved fit with reduction. Hence, the structural fractions derived from curve fitting with polylysine data may prove to be the more reliable. The relative merits of these basis spectra cannot be further resolved at this time. Both sets have been included for comparative purposes because of the uncertainties in interpretation of CD behavior, as already discussed.

In summary, it is seen that the correct native structural fractions can be produced for lysozyme by curve fitting, and this effect is useful for the present purposes. For reduced lysozyme, the greatest potential source of error appears to be from aromatic contributions, but even these may not be prohibitive for gaining qualitative insight into the nature of the denatured structure. The more significant use of CD in this work is made feasible by the capability of detecting native structure with curve fitting and has accomplished two purposes: (1) to follow the disappearance of native structure with reduction and (2) to examine the changes that might indicate a major return to native structure.

Indications from CD Studies on Lysozyme and Its Reduced Forms. The first of these purposes is fulfilled by alteration of α helix and β structure, seen with both sets, at all levels of reduction. It is clear that reduction causes a dramatic alteration in CD behavior and that there is no consistent further change from the lowest to the highest reduction level. It is already known that the fully reduced protein is greatly altered in its conformational structure (White, 1976; Imai et al., 1963).[5] Hence, by CD study, the lower reduction levels

represent a similar magnitude of alteration.

It has been shown that there is little further change when the reduced samples are subjected to the denaturing conditions necessary for carboxymethylation and ion-exchange chromatography. Moreover, the presently observed structural fractions for partially and fully reduced lysozymes are similar to those found earlier (White, 1976) for lysozyme derivatives obtained under denaturing conditions (8 M urea). Thus, removal of SS bonds under the relatively benign conditions presently used achieves this denatured state, and the presence of SS bonds therefore appears critical for maintaining the protein chain in the native conformation.

For the second purpose, the capability of detecting native structure should make it possible to see a major return of reduced lysozyme to the native state. Tamburro et al. (1970) suggested that the reduced protein under their experimental conditions exhibits a tendency toward restoration of a more ordered conformation, which is "probably the native one". There is no support from the present curve fitting results for interpretation of CD changes as indicating development of native structure in reduced lysozyme. Neither can the "qualitative similarity" seen by Saxena & Wetlaufer (1970) between CD spectra of native and reduced lysozymes be verified as an indication of native structure in the reduced state. The structural state of the reduced protein appears, rather, to be uniquely different from that of the native protein because of the disproportionation observed here and elsewhere (White, 1976; Sawyer et al., 1971; Markussen & Vølund, 1975), by which helix is lost and β structure gains on denaturation. Despite the limitations to curve fitting in its present state of development, its use would be expected to yield more meaningful information than that derived from subjective interpretation of curve shapes.

Significance of SS-Bonded Intermediates to Native Secondary Structure Development. The appearance of reduction intermediates with CD characteristics similar to those of fully reduced lysozyme indicates a high cooperativity between SS bonds and secondary structure. However, the kinetic studies that would be necessary to accumulate more information on the stepwise nature of the reductive process would be complicated by precipitation. The intermediates, trapped in this way, are not likely to be reduced further. Their relative yields may reflect the competing processes of protein precipitation and SS reduction and could be influenced by the rate of precipitation characteristic of each one.

Despite these limitations, the finding of SS-bonded intermediates in the reduction of lysozyme appears to reflect a fundamental difference between this protein and ribonuclease, for which intermediates were absent during reduction. The latter observation was made with a trapping technique by which iodoacetate served to react with molecular species containing SH groups (Creighton, 1979b). Acharya & Taniuchi (1980), also employing iodoacetate, trapped a half-reduced intermediate from the reduction of lysozyme. In addition, the present use of DTT (the same reducing agent as used for ribonuclease) resulted in a significant amount of the three-fourths-reduced intermediate (Figure 4C), together with the fully reduced product. Collectively, these findings provide strong support for the mediation of lysozyme reduction through SS-bonded intermediates, in contrast to the "all or none" effect characteristic of ribonuclease.

The appearance of intermediates in lysozyme reduction suggests that reoxidation also may proceed through SS-bonded intermediates. Acharya & Taniuchi (1978) have, in fact, isolated the three-fourths-reoxidized intermediates with all possible combinations of half-cystine residues to form native SS bonds. Further supporting this concept is the selective participation of SH groups in the reoxidation of lysozyme (Anderson & Wetlaufer, 1976).

In contrast, however, the presently observed cooperativity between SS bonds and secondary structure may suggest denaturative and regenerative mechanisms resembling those of ribonuclease (Creighton, 1977b, 1979b; Galat et al., 1981), and other proteins (Creighton, 1977a, 1979a, 1980; Creighton & Pain, 1980), with no intermediate structures.[6] Thus, for lysozyme, there exists an apparent inconsistency, with SS-bonded intermediates, but with no intermediate secondary structure detectable by CD.

The CD method, however, cannot distinguish between more subtle changes that occur within the nearly constant structural fractions. For example, the 34% β structure that characterizes the one-fourth-reduced level (Table IV) must contain more elements resembling native structure than does the equivalent fraction found for the fully reduced form (Table IV). Thus, the former protein exhibits 14% of the specific activity of native lysozyme, while no activity is detectable for the latter (Table V). Further, the extinction coefficient remains nearly native until the three-fourths-reduced level is exceeded (Figure 1 and Table II). Thus, there is ample evidence that, within these fractions, there are continued structural changes throughout the reductive process. It may be concluded, nevertheless, that a major structural change, as defined by a significant alteration of the ratio of α helix to β structure, occurs early in reduction.

It appears likely that SS-bonded intermediates produced by oxidation would also support rudiments of native secondary structure. Their relevance as essential intermediates in the further development of secondary structure remains to be established. Regardless of this question, the least likely sequence of events in chain folding of lysozyme would appear to be an extensive formation of native structure, whether thermodynamically or kinetically mediated (see introduction), followed by formation of SS bonds. The present evidence, rather, is compatible with the concept that SS bond formation is necessary prior to a major transition of secondary structure to the native state.

Acknowledgments

It is a pleasure to acknowledge the expert and diligent technical assistance of A. Gilbert Wright, Jr., throughout this work.

References

Acharya, A. S., & Taniuchi, H. (1978) *Biochemistry 17*, 3064–3070.

Acharya, A. S., & Taniuchi, H. (1980) *Int. J. Pept. Protein Res. 15*, 503–509.

[5] It may be questioned why curve fitting of CD data from a denatured protein may indicate large amounts of remaining structure, while viscosity and other physical measurements on reduced lysozyme have shown a state closer to randomness (Imai et al., 1963). An explanation comes from Lapanje (1978): "... CD spectra give information about the local chain conformation and depend very little on long range structure. Intrinsic viscosity, on the other hand, gives information about the gross conformation of the particular chain. Hence, in principle a polypeptide chain may be a random coil by the viscosity criterion, although it is still subject to particular local conformational restrictions."

[6] The findings for ribonuclease are qualified by the work of Scheraga's group. Thus, significant native structure has been found in the reduced protein (Chavez & Scheraga, 1980b). Intermediate structure is detectable during refolding (Chavez & Scheraga, 1980a; Konishi & Scheraga, 1980a). The dominant conformations in the reduced state and during refolding, however, are found to be disordered (Konishi & Scheraga, 1980a,b).

Anderson, W. L., & Wetlaufer, D. B. (1976) *J. Biol. Chem. 251*, 3147–3153.
Anfinsen, C. B. (1972) *Science (Washington, D.C.) 181*, 223–230.
Anfinsen, C. B., & Scheraga, H. A. (1975) *Adv. Protein Chem. 29*, 205–300.
Bewley, T. A. (1977) *Biochemistry 16*, 209–215, 4408–4414.
Bewley, T. A., & Li, C. H. (1969) *Int. J. Pept. Protein Res. 1*, 117–124.
Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967) *Proc. R. Soc. London, Ser. B B167*, 365–388.
Chavez, L. G., & Scheraga, H. A. (1980a) *Biochemistry 19*, 996–1004.
Chavez, L. G., & Scheraga, H. A. (1980b) *Biochemistry 19*, 1005–1012.
Chen, Y.-H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.
Creighton, T. E. (1977a) *J. Mol. Biol. 113*, 275–293.
Creighton, T. E. (1977b) *J. Mol. Biol. 113*, 329–341.
Creighton, T. E. (1979a) *J. Mol. Biol. 129*, 235–264.
Creighton, T. E. (1979b) *J. Mol. Biol. 129*, 411–431.
Creighton, T. E. (1980) *J. Mol. Biol. 137*, 61–80.
Creighton, T. E., & Pain, R. H. (1980) *J. Mol. Biol. 137*, 431–436.
Ehrenpreis, S., & Warner, R. C. (1956) *Arch. Biochem. Biophys. 61*, 38–50.
Galat, A., Creighton, T. E., Lord, R. C., & Blout, E. R. (1981) *Biochemistry 20*, 594–601.
Greenfield, N., & Fasman, G. C. (1969) *Biochemistry 8*, 4108–4116.
Habeeb, A. F. S. A. (1972) *Methods Enzymol. 25B*, 457–464.
Imai, K., Takagi, T., & Isemura, T. (1963) *J. Biochem. (Tokyo) 53*, 1–6.
Jirgensons, B. (1973) *Optical Activity of Proteins and Other Macromolecules*, Springer-Verlag, New York.
Karush, F., Klinman, N. R., & Marks, R. (1964) *Anal. Biochem. 9*, 100–114.
Katz, A., Dreyer, W. J., & Anfinsen, C. B. (1959) *J. Biol. Chem. 234*, 2897–2900.
Konishi, Y., & Scheraga, H. A. (1980a) *Biochemistry 19*, 1308–1316.
Konishi, Y., & Scheraga, H. A. (1980b) *Biochemistry 19*, 1316–1322.
Lapanje, S. (1978) *Physicochemical Aspects of Protein Denaturation*, p 34, Wiley, New York.
Leach, S. J., Minasian, E., & Reichert, L. E., Jr. (1974) *Biochim. Biophys. Acta 386*, 144–154.
Lee, C.-L., & Atassi, M. Z. (1973) *Biochemistry 12*, 2690–2695.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Markussen, J., & Vølund, A. (1975) *Int. J. Pept. Protein Res. 7*, 47–56.
Ohta, Y., Hibino, Y., Asaba, K., Sugiura, K., & Samejima, T. (1971) *Biochim. Biophys. Acta 236*, 802–805.
Sawyer, W. H., Norton, R. L., Nichol, L. W., & McKenzie, G. H. (1971) *Biochim. Biophys. Acta 243*, 19–30.
Saxena, V. P., & Wetlaufer, D. B. (1970) *Biochemistry 9*, 5015–5022.
Sears, D. W., & Beychok, S. (1973) in *Physical Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) Part C, pp 445–593, Academic Press, New York.
Shugar, D. (1952) *Biochim. Biophys. Acta 8*, 302–309.
Strickland, E. H. (1974) *CRC Crit. Rev. Biochem. 2*, 113–175.
Tamburro, A. M., Boccu, E., & Celotti, L. (1970) *Int. J. Pept. Protein Res. 2*, 157–164.
Wetlaufer, D. B., Johnson, E. R., & Clauss, L. M. (1974) in *Lysozyme* (Osserman, E. F., Canfield, R. E., & Beychok, S., Eds.) pp 269–280, Academic Press, New York.
White, F. H., Jr. (1972) *Methods Enzymol. 25B*, 387–392.
White, F. H., Jr. (1976) *Biochemistry 15*, 2906–2912.
White, F. H., Jr. (1980) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 39*, 1943.
Yutani, K., Yutani, A., Imanishi, A., & Isemura, T. (1968) *J. Biochem. (Tokyo) 64*, 449–455.