Studies on Secondary Structure in Chicken Egg-White Lysozyme after Reductive Cleavage of Disulfide Bonds†

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ABSTRACT: Native lysozyme and three reduced derivatives (carboxymethyl, carboxamidomethyl, and triphenylethylphosphonium) were examined by circular dichroism, and fractions of the protein chain present as α-helix, β structure, and unordered structure were estimated by a computer-adapted curve-fitting program. The α helix ranged from 2 to 23% in the three products, while native lysozyme exhibited 26 to 31%. However, β structure in the reduced samples occupied 23–62% of the chain length (the latter in 50% methanol), consistently in excess of the native content (11–16%). Secondary structure in the reduced samples increased with pH, while that of the native protein remained nearly constant. In 8 M urea, α helix was mostly eliminated from the reduced protein, while β structure was nearly unaffected. Qualitatively, a partial loss of β structure appeared to result from peptic digestion of the reduced samples, with further loss on exposure of the digest to urea. Stability of the observed β structure indicates its existence prior to the oxidation of SH groups, which is concomitant with development of the native conformation. This structure could, therefore, constitute at least part of a precursor conformation in the formation of native structure.

Until recently it was generally accepted that reductive cleavage of disulfide bonds would convert a protein to the randomly coiled state (White, 1961; Tanford, 1968; Anfinsen, 1973). However, with continual improvement of spectral techniques, reports have appeared of secondary structure after reduction (Yutani et al., 1968; Saxena and Wetlaufer, 1970; Ohta et al., 1971; Lee and Atassi, 1973).

A better understanding of structure that exists prior to the oxidative process of renaturation could shed more light on the mechanism by which native conformation develops. Toward this end, a CD[1] study of native lysozyme and its reduced-protected[2] derivatives was undertaken, with computer estimates for the fractions of chain length present as α helix, β structure, and unordered structure. Three reagents were used for the SH groups (iodoacetic acid, iodoacetamide, and TVP[3]), with introduction of negative, zero, and positive charges, respectively. Secondary structures in the three derivatives, as well as in native lysozyme, have been studied as functions of pH. Effects of methanol, urea, and peptic digestion on secondary structure in these proteins have also been investigated.

In addition, observations have been made in comparison of various published CD reference data used for computer analysis. The goodness of fit between the experimental and computer-determined CD curves for each sample was taken as the criterion for choice of reference data employed in this work. Thus, the data of Greenfield and Fasman (1969), which they obtained by CD examination of polylysine in the forms of α helix, β structure, and unordered structure, appeared to yield the most meaningful results in analysis of the samples described here. A preliminary report of this work has been given (White, 1976).

Experimental Section

Reduction of Lysozyme and Reaction of SH Groups. For maximal reduction and reaction of the resulting SH groups, it was found necessary to carry out these reactions twice on each sample. The procedures for reduction of lysozyme (Sigma Chemical Co., Grade I, three times crystallized) and reaction with either iodoacetic acid or iodoacetamide (Eastman Kodak Co.) were as described earlier (White, 1972).

For reaction with TVP (synthesized by the method of Schweizer and Bach (1964)), the reduced protein, after separation from the reduction mixture by precipitation with acetone–HCl and washing two times with this solvent as already described (White, 1972), was redissolved in a solution of 8 M urea containing 0.1 M triethanolamine–HCl, pH 7.9, to which a 25-fold molar excess of TVP over SH (assuming complete reduction) had been added. The reaction was allowed to proceed 30 min in a nitrogen atmosphere and the protein was precipitated and washed as before, and then redissolved in water and lyophilized. Reduction and reaction with TVP were then repeated.

The extent of reaction was estimated by phosphorus assay (Lazarus and Chou, 1972).

Specificity of the reaction was checked by amino acid analysis. The relative molar amounts of all amino acids known to be present in lysozyme (except cystine) were found within experimental error, despite the known reaction of TVP with lysine residues, which was shown to occur at higher pH values (Swan and Wright, 1971).

Amino Acid Analysis. Protein samples were acid-hydrolyzed and subjected to analysis by the earlier procedures (White and Mencken, 1970).

CD Studies. Buffer solution for all protein samples was 0.075 M sodium phosphate, pH 6.1. The solution was adjusted

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[1] Abbreviations used are: TVP, triphenylvinylphosphonium bromide; Cm-Cys, *S*-carboxymethylcysteine; Cm, carboxymethyl; Cam, carboxamidomethyl; TEP, triphenylethylphosphonium, CD, circular dichroism; ORD, optical rotatory dispersion.

[2] The term "reduced-protected" is employed throughout this paper in preference to the more commonly used term, "reduced-alkylated", and denotes chemical modification of the SH groups after reduction to prevent oxidation. Strictly speaking, the reactions of iodoacetate and iodoacetamide with SH groups are not alkylations, since alkyl groups are not introduced. Further, reaction with TVP involves only addition across a double bond.

[3] This compound is one of several potentially useful organophosphorus reagents developed by Swan and Wright (1971) and reacts with SH or amino groups by the Michael addition. Thus,

$$Ph_3P^+CH{=}CH_2 + HSR \rightarrow Ph_3P^+CH_2CH_2SR.$$

downward in pH by addition of 6 N HCl to the desired value. The possible complicating effects of chloride ions on CD behavior had been checked by addition of NaCl (maximal concentration = 0.05 M) to the phosphate buffer at the highest pH studied for each protein sample. No difference in CD curves was observed in the presence and absence of chloride.

For preparation of protein solutions, 2–5 mg of the lyophilized sample was first dissolved in water to one hundredth of the final volume; then 0.075 M sodium phosphate–HCl, at the highest pH value studied for each sample, was added to the desired level for CD study. To achieve lower pH values, each solution was adjusted downward by addition of more HCl. The volumes of HCl added were negligible and did not significantly affect the protein concentration. Attempts to dissolve the reduced samples directly in buffer led to the formation of colloidal suspensions.

For 8 M urea solutions, the urea (recrystallized as described earlier (White, 1972)) was dissolved in phosphate–HCl buffer of pH 2, and a final correction to pH 2 was made with HCl. This solution was then mixed with the concentrated protein solution in water to the desired protein concentration.

For methanol solutions, the methanol (Baker Chemical Co. Reagent Grade) was mixed with an equal volume of protein solution, and the CD study was undertaken at the resulting pH value, which was approximately one unit higher than that of the aqueous solution.

For peptic digestion, 5 mg of the protein was dissolved in 1 ml of 1% formic acid, pH 1.8, and 0.05 ml of pepsin solution (porcine, Worthington Chemical Corp., two times crystallized) at 1 mg/ml in 1% formic acid was added. Digestion continued 17 h at 37 °C. For CD study the digest was then diluted appropriately with phosphate–HCl buffer at pH 2, or with 8 M urea solution, prepared as described above.

All protein solutions were passed through Millex filters (pore size = 0.45 μm) (Millipore Corp.) prior to CD study.

Concentrations of the protein solutions ranged between 0.09 and 0.35 mg/ml and were determined with an extinction coefficient of 22.7 for $E_{1cm}^{1\%}$ at 280 nm, assumed for all reduced derivatives at the highest pH value studied for each. The coefficient had been determined after exhaustive dialysis, followed by drying at 5–10 μm of pressure over P_2O_5 at 100 °C for 17 h. The results for the three derivatives agreed within ±3%. The value for native lysozyme at 280 nm is 27.1 (Ehrenpreis and Warner, 1956).

The CD studies were carried out with a cell of 1-mm path length at 23 °C between 205 and 320 nm. The instrument used was a Cary Model 60 Spectropolarimeter with a Model 6001 CD attachment.

Treatment of the Data. The calculation of ellipticities is adequately covered elsewhere (Fasman, 1963; Jirgensons, 1973). The mean residue weights for native lysozyme, Cm-lysozyme, Cam-lysozyme, and TEP-lysozyme are 110.9, 114.6, and 128.9, respectively. When CD curves were determined in 8 M urea, the Sellmeier equation (Fasman, 1963) was used to find the refractive index correction over the range of wavelength studied. This correction was applied to the ellipticity ($[\theta]$) to determine the reduced ellipticity ($[\theta']$).

Fractions of α helix, β structure, and unordered structure were estimated by "MLAB", a curve fitting system programmed for the PDP-10 computer (Shrager, 1970). The reference curves used, representing CD behavior of the polypeptides or proteins completely in the α helical, β, or unordered forms, were chiefly the data of Greenfield and Fasman (1969), although the data of Chen et al. (1974), Chen et al. (1972), and Saxena and Wetlaufer (1971) were also tried in this system for comparative purposes.

The goodness of fit between the experimental data and the computer-determined "best fit" curve was estimated by the root mean square error defined as:

$$\sqrt{\sum \Delta\lambda^2/f} \tag{1}$$

where $\Delta\lambda$ is the difference between the experimental and best fit points at wavelength λ, and f is the number of degrees of freedom, equal to the number of measured ellipticities minus the number of unknown parameters plus the number of constraints.

This system, as applied here, employs one constraint (i.e., a condition imposed on the computation), defined as

$$A + B + C = 1 \tag{2}$$

where A, B, and C are the fractions of α helix, β structure, and unordered structure, respectively. To establish the validity of the constraint, the F test was applied to the variances resulting from computation with and without the constraint. In a typical case f_1 = number of observations (ellipticity values) − number of parameters + number of constraints = 13 − 3 + 1 = 11, when the constraint is applied. In its absence, f_2 = 10. Then

$$F = \frac{S_1^2}{S_2^2} \tag{3}$$

where the null hypothesis holds that:

$$S_1^2 = S_2^2 \tag{4}$$

The values S_1^2 and S_2^2 are the sums of the squares of all $\Delta\lambda$ values, determined with and without the constraint and divided by f_1 and f_2, respectively. From an F distribution table, the F ratio of 2.94 is exceeded 5% of the time when f_1 and f_2 = 11 and 10, respectively. With use of the curves of Greenfield and Fasman (1969) in structural analysis of native lysozyme and its reduced derivatives this value was never exceeded. Therefore, by this test the null hypothesis is valid, and presence of the constraint does not affect the fit significantly.

Results

Extent of Reduction and Reaction of SH Groups. It is essential for a study of structure after reduction to establish completeness of disulfide bond cleavage. If, for example, lysozyme were 95% reduced, as appears typical (White, 1961; Saxena and Wetlaufer, 1970), two extreme situations could obtain. First, 95% of the sample could be fully reduced with 5% containing the original four disulfide bonds/molecule. Secondly, as would seem more probable, the remaining disulfide bonds could be distributed with one bond/protein molecule, and in this event the fraction of sample so affected would be four times greater (i.e., 20 instead of 5%). The effects of a single disulfide bond on secondary structure are unknown but possibly significant, in view of the effects of tertiary constraints on CD behavior (Fasman et al., 1970; Lux et al., 1972). Thus, even the 95% reduction level would be insufficient for a study of secondary structure within a sample purportedly devoid of tertiary structure.

The present solution to this problem involves further reduction of the protein and reaction of SH groups. Table I indicates that the first reductions approximate the 95% level and are therefore inadequate for the present study. The levels of reduction, as indicated by the contents of Cm-Cys and P after the second and third steps, are higher and within experimental error identical to the theoretical values indicating full reduc-

TABLE I: Analytical Data for Reduced-Protected Lysozymes.

	Cm-lysozyme				Cam-lysozyme				TEP-lysozyme			
	½-Cystine[a]		Cm-Cys		½-Cystine		Cm-Cys		½-Cystine		Cm-Cys	
Preparation number	1	2	1	2	1	2	1	2	1	2	1	2
1st reduction	0.5	0.4	7.6	7.5	0.5	0.3	7.7	7.5	0.6	0.4	7.4	7.3
2nd reduction	0	0	8.1	8.0	0	0	8.0	7.8	0	0	7.7	7.6
3rd reduction	0	0	7.9	8.2	0	0	8.0	8.1	0	0	7.5	8.2

[a] The contents of ½-cystine, Cm-Cys, and P are expressed as mol/mol of protein.

TABLE II: Computer Analysis of Native and Reduced-Protected Lysozymes with Various Reference Data.

	Reference data	Experiment No.[a]	α Helix[b]	β Structure	Unordered structure	RMS Error[c] (deg cm^2 dmol^{-1})
Native	Chen et al. (1974)	3, 4	33, 36	8, 12	59, 52	579, 1126
	Chen et al. (1972)	3, 4	24, 27	10, 13	66, 60	719, 1310
	Saxena and Wetlaufer (1971)	3, 4	32, 37	9, 13	59, 50	439, 650
Cm	Chen et al. (1974)	13, 14	29, 28	29, 28	42, 44	908, 806
	Chen et al. (1972)	13, 14	21, 21	22, 22	57, 57	801, 912
	Saxena and Wetlaufer (1971)	13, 14	22, 21	32, 33	46, 46	901, 678
Cam	Chen et al. (1974)	23, 24	20, 20	19, 22	61, 58	436, 244
	Chen et al. (1972)	23, 24	11, 12	20, 20	69, 68	589, 188
	Saxena and Wetlaufer (1971)	23, 24	20, 18	24, 28	56, 54	331, 346
TEP	Chen et al. (1974)	33, 34	18, 24	14, 9	68, 67	854, 1203
	Chen et al. (1972)	33, 34	14, 16	32, 28	54, 56	803, 905
	Saxena and Wetlaufer (1971)	33, 34	23, 20	22, 25	55, 55	1205, 880

[a] Experiment numbers are same as those of Table III. Structural fractions and root mean square errors are given in the corresponding order on each row. [b] Structural fractions are expressed as percent of chain length. [c] Root mean square error, defined in text.

tion. The level of reduction after the second step is not increased by the third reduction. By these criteria two reductions appear sufficient for complete cleavage of the disulfide bonds.

However, the experimental error of amino acid analysis is at least ±3%, while that of the P assay, as presently carried out, is estimated at ±15–20%. The results of analysis for Cm-Cys and P, therefore, are not sufficient to establish complete removal of disulfide bonds.

Cystine analysis is a better test for completeness of reduction. This amino acid was detectable after the first reduction (Table I), but not so after the second and third even with substantial overloading of the amino acid analyzer. Thus, an amount of hydrolysate corresponding to an original protein weight of 1 mg was used, whereas one fifth this amount would suffice for good quantitation. One milligram of native lysozyme contains 0.29 μmol of cystine. The smallest measurable amount of cystine on the amino acid analyzer is taken to be 0.001 μmol. If this figure had been found after overloading, the content of disulfide bonds would have been 0.34% of the native disulfide content. Thus, we can detect 4 × 0.34 or 1.4% of the sample as a molecular species containing one disulfide bond. However, no trace of cystine was found with overloading, and the actual fraction of the sample so affected, therefore, would be less than this figure. For the present study, fractions of this order of magnitude are considered insignificant.

Choice of Reference Curves. A requirement of the computer method employed is that the dichroic behavior of α helix, β structure, and unordered structure separately must be known. There are two ways of arriving at this information. First, compounds known to be folded completely in the α helical or β conformations, or in the unordered form, may be examined directly by CD. The resulting plots of ellipticity vs. wavelength are then taken as the reference curves for these structures. Secondly, these curves may be derived mathematically from native proteins whose structures have already been elaborated by x-ray diffraction studies.

A comparison of the curves of Greenfield and Fasman (1969), which represent CD behavior of polylysine in the various conformations, with those of Chen et al. (1972), Chen et al. (1974), and Saxena and Wetlaufer (1971), all of which were derived from native proteins, indicated many differences, the possible reasons for which are adequately discussed elsewhere (Chen et al., 1972; Rosenkrantz and Scholtan, 1971; Dearborn and Wetlaufer, 1970; Cortijo et al., 1973). It is therefore obvious that the choice of reference curves is critical for the interpretation of CD behavior of an unknown sample. This choice would assume that (1) the structural moiety within the sample gives CD behavior close to or identical with that of the reference curve, and (2) that the CD behaviors of the three structural moieties are additive. Then the fractions of chain length that are involved in these structures should be proportional to their contributions to the ellipticity at a given wavelength. When these contributions are added together, the resulting value should coincide with the experimental point. In actual practice, however, such agreement is seldom observed.

A number of reasons have been given why the data of Greenfield and Fasman, in particular, might not truly represent

TABLE III: Summary of Structures in Native and Reduced-Protected Lysozymes.[a]

	Experiment No.[b]	pH	α Helix	β Structure	Unordered structure	RMS error[c] (deg cm^2 dmol^{-1})
Native	1,[d] 2	2.0	26, 27	14, 15	60, 58	625, 604
	3,[d] 4	6.1	28, 31	16, 11	56, 58	674, 825
	5,[e] 6[d,e]	6.1	31, 29	11, 11	58, 60	914, 756
Cm	7,[d,f] 8[f]	2.0	3.3, 2.2	24, 27	73, 71	404, 240
	9, 10[d]	2.0	9.6, 10	35, 32	55, 58	318, 525
	11,[d,e] 12[e]	3.3	6.3, 10	63, 54	31, 36	475, 487
	13,[d] 14	3.5	13, 15	39, 40	48, 45	587, 524
Cam	15,[f] 16[f]	2.0	0, 0	31, 33	69, 67	201, 239
	17,[d] 18	2.0	6.2, 8.5	29, 25	65, 66	432, 445
	19,[e] 20[d,e]	3.5	15, 18	62, 56	23, 26	827, 1106
	21,[d] 22	3.5	10, 12	26, 27	64, 61	475, 384
	23,[d] 24	5.1	13, 12	29, 29	58, 59	556, 425
TEP	25,[f] 26[f]	2.0	0, 0	28, 27	72, 73	322, 418
	27,[d] 28	2.0	7.8, 6.9	28, 29	64, 64	362, 478
	29,[e] 30[d,e]	4.7	23, 12	44, 45	33, 42	883, 354
	31,[d] 32	4.7	9.7, 11	25, 23	65, 66	465, 288
	33,[d] 34	5.7	9.8, 8.1	29, 33	61, 59	372, 486

[a] The reference data of Greenfield and Fasman (1969) were used throughout. [b] Odd-numbered experiments represent CD studies on preparations 1 for the three reduced derivatives (identical with those of Table I). Even-numbered experiments represent CD studies on preparations 2 of Table I. Structural compositions are expressed as percent of chain length. These figures, as well as root mean square errors, are given in the order corresponding to their experiment numbers on each row. [c] Root mean square error, defined in text. [d] The CD curves for these experiments are shown in Figures 1, 2, and 3. [e] The CD studies were performed in 50% methanol. [f] The CD studies were performed in 8 M urea.

the structures as they exist in the protein molecule (Adler et al., 1973). On the other hand, it would appear that the curves derived from native proteins should be most appropriately used in determination of structures in other native proteins. Most of the samples of this project, however, were not native, and consideration had to be given to the possibility that the CD curves of polylysine might be best suited for their study. In any case, however, the ultimate choice of reference curves would be greatly influenced by the best fit between the computed and experimental CD curves, as well as the agreement between the known structural fractions of native lysozyme and those computed with each set of reference curves.

A preliminary study was therefore undertaken to compare the results obtained with the various reference data in computation of the structural fractions of native and reduced-protected lysozymes. For native lysozyme, the data of Chen et al. (1972) produced fractions for α helix that were low in comparison with the accepted range[4] (Table II). The root mean square errors were lowest when the data of Saxena and Wetlaufer were applied (Table II). Errors associated with the data of Greenfield and Fasman (Experiments 3 and 4, Table III) were higher, although the structural fractions remained in reasonable agreement with the accepted values, as did those obtained with the data of Saxena and Wetlaufer, and that of Chen et al. (1974). However, the root mean square errors for Experiment 4 with the latter data and also that of Chen et al. (1972) were relatively large (Table II).

The data of Chen et al. (1972, 1974) and of Saxena and Wetlaufer proved less satisfactory for reduced lysozyme. Although experiments with Cam-lysozyme yielded root mean square errors from 188 to 589 (Table II), and errors associated with the data of Greenfield and Fasman in aqueous solution (Table III) were within this range, use of the other reference data (Table II) resulted in errors for Cm-lysozyme and TEP-lysozyme that were consistently above this range. Hence, the reference data representing the CD behavior of polylysine produced the lowest errors for the greatest number of experiments and, therefore, were chosen for the more detailed study of Table III.

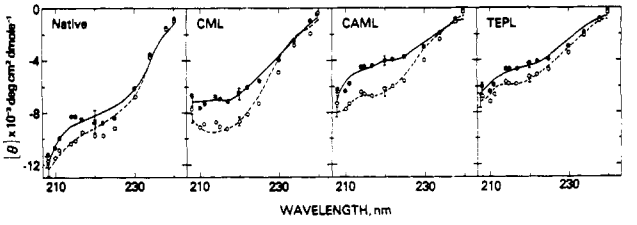

FIGURE 1: Circular dichroism of native lysozyme and its reduced derivatives in 0.075 M sodium phosphate-HCl. The curves represent experimental results at pH 2 (—) and at the higher pH studied for each sample (- - -). Closed and open circles are the computer best-fit points at the lower and higher pH values, respectively. The higher pH levels are 6.1, 3.5, 5.1, and 5.7 for native lysozyme, Cm-lysozyme (CML), Cam-lysozyme (CAML), and TEP-lysozyme (TEPL), respectively. These curves represent experiments 1 and 3 for native lysozyme, 10 and 13 for CML, 17 and 21 for CAML, and 27 and 33 for TEPL, as shown in Table III. The experiments for plotting were selected so as to represent the greatest percent of chain length in secondary structure at the lowest pH and the least structure at the highest pH. Thus, the minimal variation in CD behavior with pH is shown. Ranges indicate the extent of electronic fluctuation in the original tracings.

Examination of Native Lysozyme and Reduced Derivatives by CD in Aqueous Solution. The pH ranges for the reduced-protected proteins of Figure 1 were chosen with the lower limit at two and the upper as high as possible without causing precipitation of the protein. The three protective reagents were chosen to examine the effects of charge on the appearance of structure. Thus, the Cm, Cam, and TEP derivatives possessed negative, zero, and positive charges, respectively, introduced

[4] There is a range of uncertainty associated with the structural fractions of native lysozyme because of the difficulties in interpretation of structure derived from x-ray diffraction studies. A discussion of this problem is given by Saxena and Wetlaufer (1971). Thus, the helical content of native lysozyme lies between 28 and 42% of the chain length, while the β structure constitutes 10–16%.

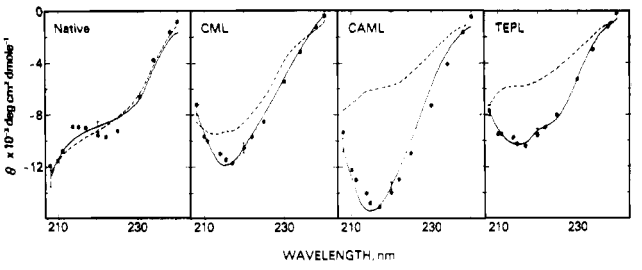

FIGURE 2: Circular dichroism of native lysozyme and its reduced derivatives in 50% methanol (—) in comparison to those in aqueous solution (- - -) at the same pH levels. Best-fit points for the samples in methanol are shown as closed circles. The curves represent Experiments 3 and 6 for native lysozyme, 11 and 13 for CML, 20 and 21 for CAML, and 30 and 31 for TEPL, as shown in Table III.

at the sulfurs. In addition, it is worth noting that TEP-lysozyme is the only derivative soluble close to pH values of physiological significance. The possible interaction between the conformational structure of TEP-lysozyme and substrate near pH 6 is a subject currently under investigation in this laboratory.

From Figure 1 it is easily seen that the least variation in CD behavior with pH occurs with TEP-lysozyme and takes place, curiously, over the widest pH range studied (2–5.7). This observation is confirmed and extended in Table III. There appears to be no consistent change in β structure with pH, with the observed changes in CD behavior resulting from a small increase in α helical content with pH. For the remaining derivatives, the variations of structure with pH are more pronounced. For Cam-lysozyme, increases in structure appear confined to α helix (Table III), while those for Cm-lysozyme involve β structure as well. At its highest pH, Cm-lysozyme exhibits the most structure seen for the three products in aqueous solution (α helix = 13–15%; β structure = 39–40%). Changes in pH have little effect on native lysozyme, probably because of the stabilizing influence of tertiary structure.

For all three derivatives, there is a preponderance of β structure in aqueous solution ranging from 23 to 40% of the total chain length, in contrast to 11–16% for the native protein. Furthermore, almost all of the experiments of Table II, with relatively large root mean square errors, also support this conclusion. The two exceptions are experiments 33 and 34, computed with the data of Chen et al. (1974) for TEP-lysozyme. Here the fractions of β structure approximate that of the native protein.

Effects of Methanol on CD Behavior. Organic solvents have frequently been reported as enhancing the formation of secondary structure in peptides, poly(amino acids), and proteins, and have this effect by suppression of ionization to raise the pK' of acidic groups to higher levels. The resulting effect on charged side chains is to reduce electrostatic repulsion and facilitate folding of the peptide chains.

In all three derivatives, the formation of secondary structure is enhanced by methanol. While Cm-lysozyme in aqueous solution at its highest pH exhibits more structure than Cam-lysozyme or TEP-lysozyme, in methanol the resulting structure is not so pronounced as for the Cam derivative (Figure 2 and Table III), to suggest that the negatively charged Cm groups hinder the formation of structure in methanol, relative to the effects of Cam groups, with zero change. The effect of methanol on native lysozyme appears negligible.

For all three derivatives, the predominant effect of methanol is to amplify the tendency toward formation of β structure.

Effects of Urea and Peptic Digestion. Results of attempts to remove secondary structure are represented in Figure 3. The

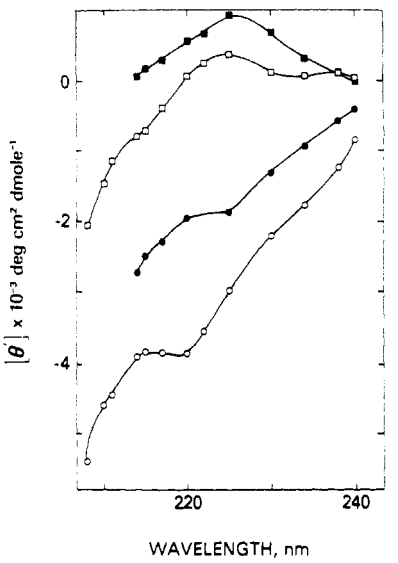

FIGURE 3: The effects of 8 M urea and peptic digestion on CD of CML. This protein, in 0.075 M phosphate-HCl (experiment 10, Table III), is represented by open circles, and in 8 M urea (experiment 7, Table III), by closed circles. Its peptic digest in phosphate-HCl is shown by open squares, and in 8 M urea by closed squares.

α helix of Cm-lysozyme is largely eliminated by 8 M urea, while β structure remains mostly intact (Table III). When this protein is digested with pepsin, there is more loss of structure as indicated by a positive shift of the entire curve, with a positive maximum for $[\theta']$ of 360 deg cm^2/dmol at 225 nm. When the digest is dissolved in 8 M urea, a further positive shift is obtained, with a maximum of +920 at 225 nm. However, computer estimates of structure in the peptic digest, whether in phosphate or urea, proved futile, with high root mean square errors and negative helical fractions. These results suggest that the remaining structure becomes altered so that the data of Greenfield and Fasman are no longer applicable.

Maxima for peptic digests of the Cam and TEP derivatives in phosphate-HCl as well as in 8 M urea occurred also at 225 nm. For Cam-lysozyme, in phosphate-HCl at pH 2, $[\theta']$ was +387 and +111, for two experiments. For TEP-lysozyme they were +934 and +889. In urea they were +951 and +848 for Cam-lysozyme; +1156 and +1040 for TEP lysozyme. Consistently, therefore, the effect of peptic digestion on the three derivatives was to produce positive maxima in phosphate-HCl which were increased by urea.

In the interpretation of these results it is important to note that the reference curves for unordered structure used in this work all show large positive ellipticities from 214 to 230 nm, while the curves for β structure show large negative values within this range. Hence shifts toward positive values, as those observed by peptic digestion and exposure to urea, suggest loss of secondary structure to produce unordered structure.

Discussion

Quantitation of CD spectra is widely regarded as a risky venture. Side chain interactions may result in structures of unpredictable CD behavior (Fasman et al., 1970). Complications may arise from variations in micropolarity about individual chromophores (Strauss et al., 1969) and from chain length effects (Chen et al., 1974). The CD approach should be regarded as affording only approximations of the true structural fractions. On the other hand, the structures found for a number of proteins are in good agreement with those known to exist from x-ray diffraction studies. Lysozyme is in

this group. Structures found for reduced lysozyme derivatives in the present work have resulted in a goodness of fit between the experimental and computer curves that is even better than that found for the native protein, suggesting that the conformational fractions found for these derivatives may be reasonably accurate. These results appear at least to offer strong support for the basic observation made in this work, which is that reduced lysozyme forms more β structure than exists in the native protein.

High levels of β structure occur in aqueous solutions as well as in 50% methanol, regardless of the charge introduced by reaction of the SH groups. The proclivity toward formation of β structure therefore appears to be a property of the reduced chain and would presumably occur in the absence of the introduced groups.

Secondary structure has been closely predicted for many native proteins on the basis of empirical rules derived by examination of a large number of native proteins whose structures had already been elaborated by x-ray diffraction studies (Chou and Fasman, 1974). For the case of a reduced protein, however, the present results suggest that disulfide bonds may prevent the chain from assuming its maximal content of β structure, possibly as dictated by the amino acid sequence, and accordingly would appear to call for some modification of the existing rules to account for the nature of the secondary structure in proteins without tertiary structure. This problem, however, is outside the scope of the present paper.

The high levels of β structure presently observed are reminiscent of the work of Markus and Karush (1957) who were probably the first to find an increased formation of secondary structure with reduction. On the basis of optical rotatory measurements, they concluded that more such structure (α helix) existed in reduced human serum albumin than in the native protein.

Aside from this early contribution the appearance of secondary structure in any form whatsoever, after removal of disulfide bonds, has only been widely recognized in recent years. It has been reported (Ohta et al., 1971; Yutani et al., 1968) that 50 and 74%, respectively, of the α helix is present after reduction of lysozyme. Both groups employed ellipticity at 222 nm as a measure of helical content, but β structure would interfere at this wavelength. Saxena and Wetlaufer (1970) found the CD curve of reduced lysozyme to be qualitatively similar to that of native lysozyme, but they made no estimate of the structural components present. Lee and Atassi (1973) reported from CD and ORD studies on two reduced-protected forms of lysozyme that they were greatly unfolded with respect to the native protein, although the presence of some secondary structure was unmistakably indicated. The presently reported excess of β structure over that in native lysozyme was not suggested by any of these studies.

This structure exhibits a stability that may appear remarkable, since it continues nearly unabated in 8 M urea and persists to some degree even after peptic digestion. However, recent observations suggest that urea may not be as effective as once believed for the production of random coils. Markussen and Volund (1975) have observed β structure for insulin and proinsulin in urea solution. Tsong (1975) has found residual structure in urea and guanidine solutions of cytochrome *c*.

The persistence of β structure is relevant to the process of renaturation. As a consequence of the nucleation hypothesis (Anfinsen, 1973), chain folding may begin prior to oxidation of the SH groups, and it has frequently been suggested that the resulting structure may resemble that of the native protein, although assumed to be greatly unfolded by comparison. However, the presently observed β structure must exist prior to oxidation because of its stability to 8 M urea, which is present during reduction as the denaturing agent. Thus, the β structure may even form simultaneously with cleavage of disulfide bonds. On separation of the reduced protein from the reduction medium, some α helix could also form, and the two structures together would constitute a precursor conformation at the start of reoxidation.

Hence, the refolding process may involve an initial incorrect formation of disulfide bonds, followed by a "shuffling", as suggested by Saxena and Wetlaufer (1970), to form the correct pairings of half-cystine residues, and at some as yet undetermined phase a loss of β structure would occur with a concomitant gain in α helix. Thus, the renaturation process, at least for lysozyme, appears to involve a more complex mechanism than that suggested by the nucleation hypothesis.

Acknowledgment

I thank Dr. Ann Ginsburg for helpful discussions and critical reading of the manuscript. Valuable information was received from Dr. Richard Hendler and Mr. Richard Shrager on the MLAB system and from Dr. Peter McPhie on circular dichroism. I am indebted to Mr. A. Gilbert Wright, Jr., for excellent technical assistance.

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Inactivation of Bovine Liver 2-Keto-4-hydroxyglutarate Aldolase by Cyanide in the Presence of Aldehydes†

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ABSTRACT: Kinetic data show that the irreversible inactivation of liver 2-keto-4-hydroxyglutarate aldolase observed when the enzyme is incubated with an aldehydic substrate (or substrate analogue) in the presence of cyanide is a biphasic process and can, under certain conditions, involve a direct interaction between the enzyme and cyanide. The kinetic data are consistent with a scheme consisting of three competing reactions: (1) irreversible addition of cyanide to the enzyme-substrate Schiff base intermediate, (2) reversible cyanohydrin formation between cyanide and the aldehydic substrate (or substrate analogue), and (3) an interaction of cyanide with the enzyme which is not substrate dependent. Approximately 0.4 mol of cyanide is associated with 1 mol (120 000 g) of enzyme when 2-keto-4-hydroxyglutarate aldolase is incubated with [^{14}C]-cyanide followed by exhaustive dialysis; an ionic attachment, possibly at a carboxylate binding site, is suggested. Whereas native enzyme, not treated with cyanide, has ten Nbs_2-titratable sulfhydryl groups, approximately one less such group reacts with Nbs_2 when the aldolase is incubated with cyanide (in the absence of aldehydic substrates). It is suggested that the binding of cyanide results in a conformational change of the enzyme; conformational changes in the presence of cyanide are confirmed by circular dichroism spectra.

L-Hydroxyproline is a major constituent of collagen. The terminal step in the catabolism of this abundant amino acid in mammals, whereby the carbon chain is degraded to smaller fragments, is catalyzed by 2-keto-4-hydroxyglutarate aldolase (2-oxo-4-hydroxyglutarate glyoxylate lyase $\rightleftharpoons$ pyruvate + glyoxylate). This enzyme, which has been highly purified from extracts of rat liver (Rosso and Adams, 1967), bovine liver (Kobes and Dekker, 1969), and *Escherichia coli* (Nishihara and Dekker, 1972), catalyzes the dealdolization of kHOGlt,[1] yielding equimolar amounts of pyruvate and glyoxylate. Although the reaction is readily reversible, currently available information appears to indicate that the primary role of this aldolase in mammals is to catalyze kHOGlt degradation rather than its synthesis.

kHOGlt-aldolase from bovine liver is a Schiff base mechanism (class I) aldolase with a molecular weight of 120 000 (Kobes and Dekker, 1969, 1971a). As an aldolase, it is unusual in that it (a) binds not only kHOGlt and pyruvate but also glyoxylate via an azomethine linkage to the ϵ-amino group of an active-site lysyl residue (Kobes and Dekker, 1966, 1971a); (b) nonstereospecifically catalyzes the cleavage or formation of the D and L isomers of kHOGlt (Kobes and Dekker, 1971b); and (c) is 50% as effective as a β-decarboxylase toward oxalacetate as it is as an aldolase with kHOGlt (Kobes and Dekker, 1971b).

Cyanide is, for a variety of reasons, a versatile inhibitor of enzyme-catalyzed reactions; it readily combines with the metal ion which is essential for the activity of certain enzymes (i.e., cytochrome oxidase), it forms a cyanohydrin with carbonyl compounds that are required for enzyme activity (i.e., pyridoxal phosphate requiring enzymes), it causes slow but virtually irreversible inactivation of some enzymes by scission of essential disulfide linkages, and it also destroys xanthine oxidase activity by elimination of sulfur as thiocyanate from the protein (Massey and Edmondson, 1970). We previously reported that cyanide causes a substrate-dependent irreversible inactivation of kHOGlt-aldolase; no loss of activity is observed when the enzyme is incubated with kHOGlt or pyruvate in the presence of cyanide, but with glyoxylate (or other aldehydes, notably formaldehyde, glyoxal, or glycolaldehyde) a very rapid and irreversible loss of aldolase activity occurs (Kobes and Dekker, 1967; Hansen, et al., 1974). Working with either [^{14}C]cyanide or a ^{14}C-labeled aldehyde, we recently showed

† From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109. *Received December 22, 1975.* This investigation was supported by Grant AM-03718 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, United States Public Health Service.

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[1] Abbreviations used are: kHOGlt, 2-keto-4-hydroxyglutarate; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); CD, circular dichroism; LDH, lactate dehydrogenase; DEAE, diethylaminoethyl; NADH, reduced nicotinamide adenine dinucleotide.