Halper, J. P., Latovitzki, N., Bernstein, H., and Beychok, S. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 517.
Hayashi, K., Imoto, T., and Funatsu, M. (1964), *J. Biochem. (Tokyo) 55*, 516.
Herskovits, T. T., and Laskowski, M. (1962), *J. Biol. Chem. 237*, 2481.
Herskovits, T. T., and Sorensen, M. (1968), *Biochemistry 7*, 2523.
Ikeda, K., and Hamaguchi, K. (1970), *J. Biochem. (Tokyo) 68*, 785.
Ikeda, K., Hamaguchi, K., Miwa, S., and Nishina, T. (1972), *J. Biochem. (Tokyo) 71*, 371.
Imoto, T., Forster, L. S., Rupley, J. A., and Tanaka, F. (1971), *Proc. Nat. Acad. Sci. U. S. 69*, 1151.
Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C., and Rupley, J. A. (1972), *Enzymes 7*, 665.
Isaka, K., Shirakawa, H., Yamada, M., and Suyama, T. (1971), *Anal. Biochem. 42*, 299.
Jolles, P., Charlemagne, D., Dianoux, A. C., Jolles, J., Le Baron, J. L., and Saint-Blancard, J. (1968), *Biochim. Biophys. Acta 151*, 532.
Jolles, J., and Jolles, P. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 22*, 31.
Kronman, M. J., Holmes, L. G., and Robbins, F. M. (1971), *J. Biol. Chem. 246*, 1909.
Kuramitsu, S., Ikeda, K., Hamaguchi, K., Miwa, S., and Nishina, T. (1972), *J. Biochem. (Tokyo) 72*, 1109.
Latrovitzki, N., Halper, J. P., and Beychok, S. (1971), *J. Biol. Chem. 264*, 1457.
Lehrer, S. S., and Fasman, G. D. (1966), *Biochem. Biophys. Res. Commun. 23*, 133.
Lehrer, S. S., and Fasman, G. D. (1967), *J. Biol. Chem. 242*, 4644.
Mouton, A., and Jolles, J. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4*, 337.
Osserman, E. F., and Lawlor, D. P. (1966), *J. Exp. Med. 124*, 921.
Raftery, M. A. (1969), *Anal. Biochem. 30*, 427.
Rupley, J. A. (1964), *Biochim. Biophys. Acta 83*, 245.
Rupley, J. A., and Gates, V. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 496.
Sharon, N., and Seifter, S. (1964), *J. Biol. Chem. 239*, 2398.
Teichberg, V. I., Plasse, T., Sorell, S., and Sharon, N. (1972), *Biochim. Biophys. Acta 278*, 250.
Teichberg, V. I., and Sharon, N. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 171.

Conformation and Immunochemistry of Methylated and Carboxymethylated Derivatives of Lysozyme†

Ching-Li Lee and M. Z. Atassi*

ABSTRACT: Reduction of the disulfide bonds of lysozyme with 2-mercaptoethanol in 8 M urea followed by reaction with iodoacetic acid or with methyl *p*-nitrobenzenesulfonate gave two derivatives. One derivative was carboxymethylated (SCM-lysozyme[1]) and the second was methylated (SM-lysozyme) at the eight cysteine residues resulting from reduction of the four disulfide bonds. Optical rotatory dispersion (ORD) and circular dichroism (CD) measurements in water showed that the two derivatives were greatly unfolded relative to native lysozyme. There was some indication from CD measurements that SM-lysozyme was somewhat more folded than SCM-lysozyme in water. Conformational studies in increasing concentrations of methanol suggested that SM-lysozyme assumed some structural stability around 35% methanol while SCM-lysozyme showed no discreetly stabilized structure in the range 0–60% methanol. The stabilized structure of SCM-lysozyme had ORD parameters that approximated those of native lysozyme. Further confirmation of this was obtained from immunochemical studies. SCM-lysozyme showed no reaction (0%) with antisera to lysozyme. On the other hand, SM-lysozyme showed appreciable (38%) cross-reaction with these antisera. However, the enzymic activity in each derivative was completely eliminated, suggesting that more rigid structural requirements are needed for this property than for immunochemical cross-reaction. The present findings indicated that it was indeed feasible, at least to a limited extent, to effect a stabilized structure in SM-lysozyme due to the ability of the *S*-methyl groups to participate in nonpolar interactions. In SCM-lysozyme, the directive effect of long-range interactions is ineffective because a refolded, stabilized structure is prevented by steric effects and by the like-charge repulsion between the carboxymethyl anions as they approach one another.

It is now well established that protein conformation is a direct consequence of primary structure (Lumry and Eyring, 1954; Sela *et al.*, 1957; White and Anfinsen, 1959; Anfinsen, 1961, 1964; White, 1960). The major role in stabilizing protein folding is contributed by long-range interactions (Singhal and Atassi, 1970; Atassi and Singhal, 1970), since an intact whole

† From the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received February 23, 1973*. This paper is the sixth in the series: Conformational Studies on Modified Proteins and Peptides. This work was supported by the American Heart Association (Grant No. 71-910) and in part by a grant (AM-13389) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

[1] The abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: SCM-lysozyme, a derivative of lysozyme in which the disulfide bonds were reduced and then the resulting cysteine residues were carboxymethylated by reaction with iodoacetic acid; SM-lysozyme, a lysozyme derivative obtained by reduction of the disulfide bonds followed by methylation with the use of methyl *p*-nitrobenzenesulfonate.

helix in the protein is less helical in the free state (Singhal and Atassi, 1970) and helicity increases as probability of stabilization by long-range interactions improves (Singhal and Atassi, 1970). In addition, many proteins contain disulfide bonds. The cross-links effected by disulfide bonds provide covalently stabilized structures. Disulfide bonds, ruptured by reduction, will re-form correctly on reoxidation (Sela *et al.*, 1957; White and Anfinsen, 1959; Anfinsen, 1961, 1964; White, 1960) pointing to the critical directive effect exerted by long-range interactions. However, when the disulfide bonds are cleaved by oxidation (Sanger, 1949; Ryle *et al.*, 1955; Hirs, 1956; Redfield and Anfinsen, 1956) or by reduction followed by carboxymethylation (White, 1960; Anfinsen and Haber, 1961; Crestfield *et al.*, 1963), the native three-dimensional structure of the protein is completely disrupted in spite of the directive force of long-range interactions. Clearly, in these derivatives, satisfactory approach of the previously covalently linked regions is prevented by like-charge repulsion. It is interesting to point out here that no work has been done to investigate the possibility of improving the reapproach of regions previously linked by disulfide bonds and hence achieve a better approximation of the native three-dimensional structure. This may be feasible by eliminating the like–like charge repulsion (encountered on employing the foregoing scission procedures) without introducing the steric hindrance of a bulky side chain. Such a concept was examined in the present work using lysozyme, which has four disulfide bonds (Canfield and Liu, 1965a,b; Blake *et al.*, 1965), as the protein model. Two derivatives were prepared, one in which the disulfides were ruptured by reduction followed by carboxymethylation and in the other, reduction of the disulfides was followed by methylation (Heinrikson, 1970). The conformations of the two derivatives were investigated in detail by ORD and CD measurements. Also, extent of attainment of the native three-dimensional structure was examined independently by studying the immunochemistry and enzymic activity of the derivatives.

Experimental Section

Materials. Lysozyme (three-times crystallized) was purchased from Sigma Chemical Co. and was found to be homogeneous by starch gel, acrylamide gel, and disc gel electrophoresis. Methyl *p*-nitrobenzenesulfonate was obtained from Pierce Chemical Co. 2-mercaptoethanol was from Eastman Chemical Co. Iodoacetic acid was obtained from Sigma Chemical Co. *Micrococcus lysodeikticus* cells were freeze-dried vials from Worthington Biochemical Corp. All other chemicals employed were of analytical grade.

Reduction and S-Carboxymethylation of Lysozyme. To lysozyme (100 mg; 6.99 μmol), dissolved in 8 M urea in 0.25 M triethylamine acetate buffer (pH 8.2, 5 ml), 2-mercaptoethanol (0.5 ml) was added. The mixture was incubated under nitrogen for 4 hr at 37° with continuous mixing. Reduction was terminated by precipitation of the protein with 40 ml of ethanol–HCl (2% HCl in 98% ethanol). After standing under nitrogen at −20° for 3 hr, it was centrifuged (1800 rpm for 40 min) and washed with ethanol–HCl (200 ml) until freed of 2-mercaptoethanol (about 4 times). During centrifugation, the liquid was under nitrogen in capped centrifuge bottles. The protein was then dissolved in 8 M urea at pH 8.2 (5 ml) containing iodoacetic acid (100 mg). Reaction mixture was kept in the dark, under nitrogen and maintained at pH 8.2 for 2 hr by the addition of 0.5 N NH_4OH on the pH-Stat at room temperature. Excess iodoacetic acid was destroyed by the addition of 2-mercaptoethanol (0.5 ml) to the solution at pH 8.2. The *S*-carboxymethyllysozyme was precipitated with ethanol–HCl (40 ml), washed on the centrifuge twice with ethanol–HCl, suspended in water, dialyzed against four 2-l. changes of distilled water and freeze-dried. The completeness of reduction and carboxymethylation and the specificity of the latter were confirmed by amino acid analysis.

TABLE I: Molecular Weights, Mean Residue Weights, and Nitrogen Contents of Lysozyme and Derivatives.[a]

Protein	Mol Wt	M_R	N Content (%)
Lysozyme	14,307	110.90	18.80
SCM-lysozyme	14,779	114.57	18.20
SM-lysozyme	14,427	111.84	18.64

[a] Values were calculated from the amino acid composition of lysozyme and its derivatives.

Reduction and S-Methylation of Lysozyme. Lysozyme (200 mg; 13.98 μmol) was dissolved in 8 M urea (15 ml) in Tris buffer at pH 8.6 (6.973 g of Tris, 12 ml of 1 N HCl, and 4 ml of 5% EDTA diluted to 100 ml) in a brown bottle under nitrogen. 2-Mercaptoethanol (100 μl) was added and the solution was stirred magnetically under nitrogen at room temperature for 2 hr. The temperature was then elevated to 40° and a solution (2 ml) of methyl *p*-nitrobenzenesulfonate (434 mg; 2 mmol) in dioxane was added dropwise to the magnetically stirred solution under nitrogen. After reaction at 40° for 30 min, the reaction mixture was passed through a Sephadex G-10 column (3.0 × 40 cm) which was eluted with 8 M urea to remove excess reagent. Tubes containing the protein fraction were combined, dialyzed extensively against distilled water, and then freeze-dried. The completeness of reduction and S-methylation and specificity of the latter were confirmed by amino acid analysis.

Analytical Methods. Starch gel electrophoresis was carried out in 0.02 M phosphate buffer at pH 7.5, in 0.1 M formic acid at pH 2.6, or in 4 M urea prepared in 0.02 M Na_2HPO_4 (pH 8.9). Procedures for starch gel electrophoresis have been described elsewhere (Atassi and Caruso, 1968). Amino acid analyses of protein hydrolysates were carried out on a BioCal BC-200 amino acid analyzer. Acid hydrolysis was in constant-boiling HCl (double distilled) at 110° for 22 hr, or 72 hr in nitrogen-flushed evacuated sealed tubes. For tryptophan determination, hydrolysis was in 3 N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Liu and Chang, 1971) in nitrogen-flushed, evacuated sealed tubes at 110° for 22 or 48 hr.

Concentrations of protein solutions were based on their nitrogen contents which were calculated from their amino acid compositions. Table I shows the molecular weights, nitrogen contents and mean residue weights for lysozyme and the present two derivatives. Analyses for nitrogen were done by a micro-Kjeldahl procedure (Markham, 1942) and by using Nessler's reagent standardized with ammonium sulfate. Three or more replicate analyses were usually performed on each solution and the results varied ±0.5% or less.

Enzymic activity was determined in 0.06 M phosphate buffer, containing 0.09% NaCl (pH 6.2) from the rate of lysis of *M. lysodeikticus* as described elsewhere (Atassi and Habeeb, 1969). Also, in the present work, enzymic activity was determined in 0.06 M phosphate, containing 0.09% NaCl (pH 6.2) and which was 35% with respect to methanol.

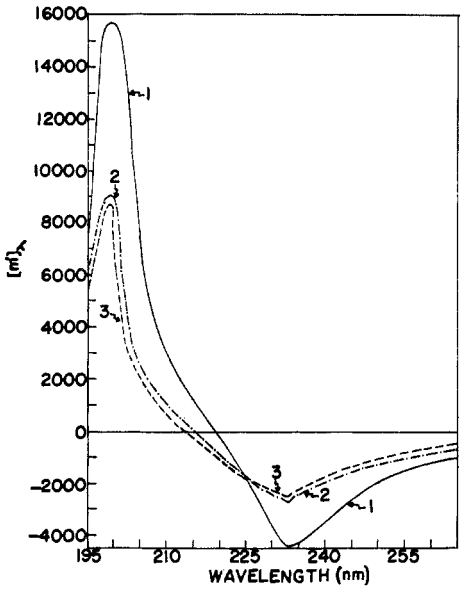

FIGURE 1: ORD spectra of Lysozyme and derivatives in water: 1, lysozyme; 2, SM-lysozyme; 3, SCM-lysozyme.

Optical Rotatory Dispersion and Circular Dichroism Measurements. ORD and CD studies were carried out at 25° on solutions of lysozyme or its derivatives in water. Solutions contained 0.05–0.2 mg of protein/ml. Measurements were made with a Cary Model 60 spectropolarimeter, equipped with a Model 6001 circular dichroism accessory. Each sample was measured at several concentrations employing cells with light paths of 0.5, 1, 5, and 10 mm. Each sample was scanned at least three times at each concentration and the ORD and CD parameters were the average from these scans. ORD data are reported in reduced mean residue rotation $[m']_\lambda$ corrected for the refractive index dispersion of water, n_λ. The Moffitt–Yang parameter, b_0, was calculated from their equation (Moffitt and Yang, 1956) with λ_0 as 212 nm. CD data are presented here in reduced molar ellipticities $[\theta']_\lambda$, corrected for the refractive index dispersion of water, n_λ (*i.e.*, $[\theta'] = [\theta]3/(n^2 + 2)$). Units of $[\theta']$ are in (deg cm^2) per dmol. Experimental procedure and quantitative treatment of ORD and CD data have been described elsewhere (Atassi and Singhal, 1970).

Effect of methanol on conformation of lysozyme and its present two derivatives was studied by the addition of increasing amounts of methanol to a solution of lysozyme, or derivative, in water. After each methanol addition, the protein solution was centrifuged (4000 rpm, 1 hr, 0°) and aliquots were then removed for triplicate nitrogen analyses. Therefore, the exact protein concentration at each methanol content was known and complications due to any precipitation of protein on addition of methanol were completely avoided.

Immunochemical Methods. Preparation of goat antisera to lysozyme has already been described (Atassi and Habeeb, 1969). For the present work, two goat antisera, G9 and G10, were used. The sera were kept separate and stored in 5-ml aliquots at −40°. Quantitative precipitin and absorption experiments were performed as described elsewhere (Atassi and Saplin, 1968).

Results

Characterization of the Derivatives. On starch gel electrophoresis at pH 7.5, SCM-lysozyme and SM-lysozyme behaved in a similar manner to native lysozyme. On starch gel electrophoresis at pH 2.6, SCM-lysozyme showed a single, positively charged band that moved faster than native lysozyme with mobility of 1.43 (relative to native lysozyme = 1), but SM-lysozyme moved slower than native lysozyme with mobility of 0.78. Finally in electrophoresis at pH 8.9 in urea, SCM-lysozyme did not migrate from the origin, while SM-lysozyme showed mobility = 0.57 (relative to native lysozyme = 1). Each derivative was completely homogeneous in electrophoresis at each pH value and in neither derivative was there any trace of unmodified lysozyme present.

TABLE II: Amino Acid Composition of Lysozyme and Derivatives.[a]

Amino Acid	Lysozyme	SCM-Lysozyme	SM-Lysozyme
S-CM-cysteine	0.00	7.89	0.00
Asp	20.9	20.6	20.6
Thr	6.70	6.51	7.05
Ser	9.76	9.70	9.71
Glu	5.15	5.21	5.07
Pro	2.01	1.97	2.00
Gly	12.00	11.96	12.24
Ala	12.12	12.14	12.18
Cys	7.90	0.00	0.00
Val	5.86	5.76	5.97
Met	2.03	2.04	1.96
Ile	5.69	5.72	5.62
Leu	8.10	8.11	7.70
Tyr	2.95	3.19	2.69
Phe	2.96	3.14	2.83
Trp	5.88	5.98	5.78
Lys	6.13	5.82	5.72
His	1.02	1.31	1.05
Arg	11.10	10.81	11.25
S-Methylcysteine[b]	0.00	0.00	8.01

[a] The results represent the average of four acid hydrolyses (two at 22 hr and two at 72 hr) and duplicate hydrolyses with *p*-toluenesulfonic acid. The values for threonine and serine have been obtained by extrapolation to zero hydrolysis time. Tryptophan was determined from the *p*-toluenesulfonic acid hydrolysis. [b] The constant for *S*-methylcysteine was 90% of the value for alanine (Heinrikson, 1970).

Amino acid analysis of native lysozyme, carboxymethylated lysozyme and S-methylated lysozyme are presented in Table II. Amino acid analysis showed complete modification of the 8 cysteine residues (obtained after reduction with mercaptoethanol) by carboxymethylation with iodoacetic acid, or by methylation with methyl *p*-nitrobenzenesulfonate. No other amino acids were modified. From amino acid composition data, it was concluded that cleavage and modification had taken place specifically at the disulfide bonds.

Optical Rotatory Dispersion Measurements. In ORD measurements, lysozyme and its derivatives showed negative rotation with a minimum at 233 nm and a positive rotation maximum at 199 nm. Figure 1 shows the ORD spectra of lysozyme and its derivatives in the ultraviolet region. Lysozyme showed the highest rotation both at the negative minimum at 233 nm and at the positive extremum at 199 nm (Table III). SM-lysozyme showed a decrease in its rotation both at $[m']_{233}$ and $[m']_{199}$. SCM-lysozyme was also less rotatory than

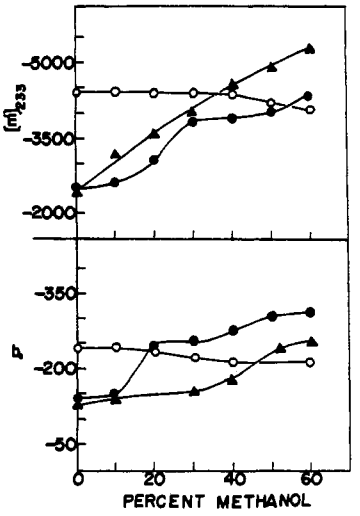

FIGURE 2: Effect of methanol on the rotatory behavior of lysozyme and its derivatives. The *upper part* shows change of $[m']_{233}$ and the *lower part* shows change of b_0 with increasing methanol concentration: (○) lysozyme; (●) SM-lysozyme; (▲) SCM-lysozyme.

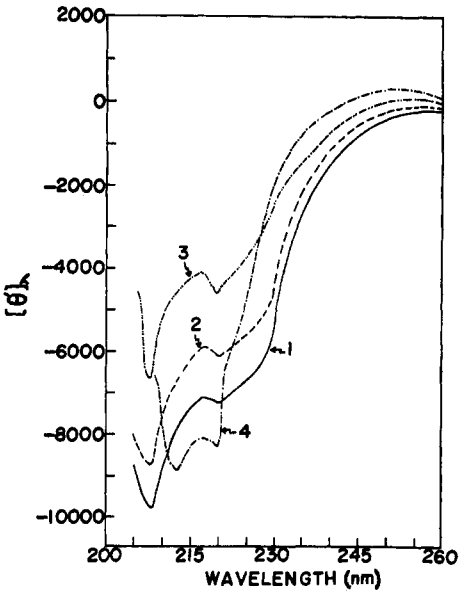

FIGURE 3: CD spectra of: 1, lysozyme in water; 2, lysozyme in 60% methanol; 3, SCM-lysozyme in water; 4, SCM-lysozyme in 60% methanol.

lysozyme at the negative minimum and at the positive extremum (Table III).

The effect of methanol on the rotatory behavior of lysozyme and its two derivatives was investigated. Figure 2 shows the variations of $[m']_{233}$ with methanol concentration. For SCM-lysozyme and SM-lysozyme, the rotatory power was increased on addition of methanol. The increased rotatory power in the presence of methanol was also accompanied by increase in the b_0 values (see Figure 2). However, the curves for these increases in SM-lysozyme showed distinct shoulders between 30 and 40% methanol. Table III summarizes the values of $[m']_{233}$, $[m']_{199}$, and b_0 for each of these derivatives in water and 60% methanol (v/v). Lysozyme showed some slight decrease in the $[m']_{233}$ and the b_0 values (Figure 2) on addition of methanol.

Circular Dichroism Measurements. Lysozyme showed negative ellipticity bands at 220 and 208 nm both in water and in 60% methanol. On the other hand, SCM-lysozyme and SM-lysozyme showed negative ellipticity bands, in 60% methanol, at 220 and 212–213 nm. The ellipticity values for SCM-lysozyme and SM-lysozyme were also increased in the presence of methanol. For lysozyme, however, a slight decrease in ellipticity was observed (Figure 3 and Table IV). Figure 3 also shows the circular dichroism behavior of SCM-lysozyme in water and in 60% methanol (v/v). In 60% methanol, the $[\theta']$ value for SCM-lysozyme at 220 nm was almost twice the corresponding value in water. The negative ellipticity band at 212–213 nm in 60% methanol was also appreciably higher than the ellipticity band at 208 nm in water (Table IV). The circular dichroism behavior of SM-lysozyme is shown in Figure 4. SM-lysozyme showed negative ellipticity which, in water, appeared at 220 and 208 nm and in 60% methanol at 220 and 212–213 nm, and these bands were also increased in the presence of methanol (see Table IV).

Immunochemistry of the Derivatives. In quantitative precipitin analysis with antisera to lysozyme, SCM-lysozyme and SM-lysozyme behaved differently. Table V summarizes the results of the precipitin reactions of SCM-lysozyme and SM-lysozyme with goat antisera to lysozyme, G9 and G10. SCM-lysozyme failed to react with antisera to lysozyme. On the other hand, SM-lysozyme showed substantial (35–38%) reactivity relative to the homologous reaction. Also, SM-lysozyme absorbed 38% of the reactivity of antisera to lysozyme with the homologous antigen (*i.e.*, lysozyme).

Enzymic Activity of Lysozyme and Its Derivatives. Reduction of cystine residues of lysozyme followed by carboxymethylation or by methylation, resulted in complete (100%) loss of enzymic activity relative to native lysozyme. In addition, enzymic activity was determined in 0.06 M phosphate buffer containing 0.09% NaCl (pH 6.2) and which was 35% with respect to methanol. Whereas lysozyme possessed full lytic activity in this solvent, neither SCM-lysozyme nor SM-lysozyme displayed any enzymic activity.

TABLE III: Optical Rotatory Dispersion Parameters of Lysozyme and Derivatives in Water and in 60% Methanol.

Protein	Solvent	$[m']_{233}$	b_0
Lysozyme	Water	−4380	−240
	60% Methanol	−4020	−210
SCM-lysozyme	Water	−2450	−133
	60% Methanol	−5230	−245
SM-lysozyme	Water	−2540	−145
	60% Methanol	−4140	−286

TABLE IV: Circular Dichroism Parameters of Lysozyme and Its Derivatives in Water and in 60% Methanol.

Protein	Solvent	$[\theta']_{220}$	$[\theta']_{208}$
Lysozyme	Water	−7210	−9740
	60% Methanol	−6000	−8700
SCM-lysozyme	Water	−4530	−6640
	60% Methanol	−8220	−8840[a]
SM-lysozyme	Water	−5840	−7860
	60% Methanol	−7670	−8150[a]

[a] These bands appeared at 212.5 nm.

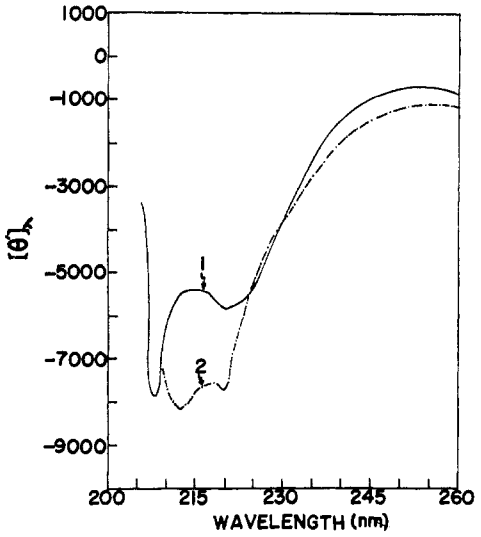

FIGURE 4: CD spectra of SM-lysozyme in (1) water and (2) 60% methanol.

Discussion

The relatively high pH conditions employed in the present reactions may be expected to give rise to carboxymethylation or methylation of histidine or lysine residues. However, the results of amino acid analysis ruled out this possibility completely, since these adducts of lysine or histidine will be stable to acid hydrolysis. Furthermore, the gel electrophoresis results demonstrated the complete homogeneity of the two derivatives, and also no trace of unmodified lysozyme was detected in either SCM-lysozyme or SM-lysozyme. Therefore, the present conformational and immunochemical differences between SCM-lysozyme and SM-lysozyme could not have been caused by heterogeneity of the derivatives or contamination with native protein.

From ORD measurements in water, it was clear that SM-lysozyme, like SCM-lysozyme, was greatly unfolded relative to native lysozyme. There was essentially no difference in the $[m']_{233}$ and $[m']_{199}$ values of the two derivatives. However, the difference in the b_0 values, which was outside the range of the normal experimental variation, suggested that SM-lysozyme may be somewhat more folded than SCM-lysozyme in water. This conclusion was more clearly evident from the results of CD measurements on the two derivatives in water. The values of $[\theta']_{220}$ and $[\theta']_{208}$ for SM-lysozyme were about 20% higher than the corresponding values for SCM-lysozyme. If SM-lysozyme, indeed, had a tendency to be more folded in water than SCM-lysozyme, then it was of interest to test the feasibility of achieving a more stabilized SM-lysozyme conformation. The results of ORD measurements in varying concentrations of methanol were highly significant. Whereas methanol exerted a very slight effect on the rotatory power and ellipticity values of native lysozyme, its effect on these parameters for the two derivatives differed markedly. The steady increase of the ORD and CD parameters of SCM-lysozyme reflected no conformational state of this derivative was particularly stabilized. The increase in methanol concentration most likely favored progressive aggregation which accounted for the steady increase of the ORD and CD parameters. In contrast, the values of $[m']_{233}$ and b_0 for SM-lysozyme, which did not increase steadily but showed a definite shoulder between 30 and 40% methanol, indicated a stabilized structure whose parameters were very similar to those of native lysozyme. Above 40% methanol, this stabilized structure was disrupted. At low methanol concentration intramolecular long-range interactions may be favored, but above 40%, methanol intermolecular interactions become dominant and induce aggregation which will be apparent in further increase of the ORD and CD parameters of the derivative.

TABLE V: Comparison of Antigenic Reactivities of Lysozyme, SCM-Lysozyme, and SM-Lysozyme.[a]

Protein	Antiserum G 9	Antiserum G 10
Lysozyme	100	100
SCM-lysozyme	0	0
SM-lysozyme	35	38

[a] Values are in per cent precipitation relative to homologous antigen and represent the average of three determinations which varied ±1.0% or less.

The foregoing conclusions, suggesting a stabilized more folded structure of SM-lysozyme, which was derived from ORD and CD measurements, made it intriguing to test the biological properties of the derivative. If the native conformation of lysozyme was somewhat approximated in SM-lysozyme, then it appeared likely that the latter may exhibit some of the biological properties of the native protein. However, SM-lysozyme behaved like SCM-lysozyme with respect to enzymic activity. Even at 35% methanol, where SM-lysozyme achieved a stabilized conformation similar in parameters to those of the native protein, this derivative still possessed no enzymic activity. At this stage, the findings may have appeared contradictory, and it was decided to monitor another biological property, namely the immunochemistry of the derivatives.

It is now well established that the antigenic reactivity of native protein antigens is highly influenced by changes in their conformation (Atassi, 1967; Habeeb, 1967; Atassi and Skalski, 1969; Andres and Atassi, 1970). Furthermore, the primary antibody response is directed against the native, three-dimensional structure of a protein antigen (Atassi and Thomas, 1969). Accordingly, immunochemical methods may be employed, under appropriate conditions, as a powerful and highly sensitive tool to monitor conformational changes in proteins (Atassi, 1967). However, it may be relevant to caution here that not every conformational change will exert an effect on the antigenic reactivity (Atassi and Habeeb, 1969; Atassi *et al.*, 1971, 1972). This will be dependent on the protein and the nature of the conformational reorganization. With antisera to native lysozyme, SCM-lysozyme showed no reaction confirming the presence of a drastic conformational change in this derivative. This lack of immunochemical cross-reaction obtained with SCM-lysozyme was not unexpected and has recently been reported by other workers (Shinka *et al.*, 1967; Gerwing and Thompson, 1968; Young and Leung, 1970). Similarly, it had been observed that rupture of the disulfide bonds in ribonuclease resulted in complete elimination of its antigenic reactivity with antisera to ribonuclease (Brown, 1962). In view of these reports and the present findings on SCM-lysozyme, the substantial (38%) antigenic reactivity of SM-lysozyme observed here must be considered as very significant indeed. Certainly, the cross-reaction of SM-lysozyme was not caused by contamination with native lysozyme. Immunochemical cross-reaction of proteins is a function of

similarity in sequence of antigenic reactive regions and also close conformations of these regions are required for appropriate fit onto the antibody combining site (Atassi, 1970; Atassi *et al.*, 1970; Habeeb and Atassi, 1971). The present findings can best be explained by concluding that the immunochemically unreactive SCM-lysozyme undergoes a complete change in mode of folding so that it will retain no favorably folded reactive regions that will correctly fit into the antibody combining site. On the other hand, SM-lysozyme must still exhibit partial retention of the native conformation, at least in some parts of the molecule, making it possible for some reactive regions to achieve a configuration that may bind with antibody.

The present findings, therefore, clearly show that long-range interactions exert a considerable driving force for reorganizing the molecule into its native conformation. In SCM-lysozyme (or when the disulfides are oxidized to cysteic acid), the directive effect of long-range interactions cannot give rise to a structure which resembles that of the native protein because this will be prevented by steric effects and by the like–like charge electrostatic repulsion between the carboxymethyl anions as they approach one another. By contrast in SM-lysozyme, the *S*-methyl groups are smaller and they may participate in hydrophobic interactions and thus induce stabilization of the SM-lysozyme conformation, or at least certain regions of it. The lack of enzymic activity of SM-lysozyme demonstrates that full recovery of the native conformation was not achieved. For this property, where many amino acid side chains are required to make contact with the substrate (Blake *et al.*, 1965), more stringent conformational features are required than those needed for antigenic reactivity. In the latter, if certain regions achieve the proper conformation, they may bind with their respective antibodies even though other antigenic regions on the molecule are improperly folded and remain unreactive.

References

Andres, S. F., and Atassi, M. Z. (1970), *Biochemistry 9*, 2268.

Anfinsen, C. B. (1961), *J. Polym. Sci. 49*, 31.

Anfinsen, C. B. (1964), *in* New Prospectives in Biology, Sela, M., Ed., Amsterdam, Elsevier, p 42.

Anfinsen, C. B., and Haber, E. (1961), *J. Biol. Chem. 236*, 1361.

Atassi, M. Z. (1967), *Biochem. J. 103*, 29.

Atassi, M. Z. (1970), *Biochim. Biophys. Acta 221*, 612.

Atassi, M. Z., and Caruso, D. R. (1968), *Biochemistry 7*, 699.

Atassi, M. Z., and Habeeb, A. F. S. A. (1969), *Biochemistry 8*, 1385.

Atassi, M. Z., Perlstein, M. T., and Habeeb, A. F. S. A. (1971), *J. Biol. Chem. 246*, 3291.

Atassi, M. Z., and Saplin, B. J. (1968), *Biochemistry 7*, 688.

Atassi, M. Z., and Singhal, R. P. (1970), *J. Biol. Chem. 245*, 5122.

Atassi, M. Z., and Skalski, D. J. (1969), *Immunochemistry 6*, 25.

Atassi, M. Z., Suliman, A. M., and Habeeb, A. F. S. A. (1972), *Immunochemistry 9*, 907.

Atassi, M. Z., Tarlowski, D. P., and Paull, J. H. (1970), *Biochim. Biophys. Acta 221*, 623.

Atassi, M. Z., and Thomas, A. V. (1969), *Biochemistry 9*, 3385.

Blake, C. C. F., Koenig, D. F., Mair, G. A., North, A. C. T., Philips, D. C., and Sarma, V. R. (1965), *Nature (London) 206*, 757.

Brown, R. K. (1962), *J. Biol. Chem. 237*, 1162.

Canfield, R. E., and Liu, A. K. (1965a), *J. Biol. Chem. 240*, 1998.

Canfield, R. E., and Liu, A. K. (1965b), *J. Biol. Chem. 240*, 2002.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Gerwing, J., and Thompson, K. (1968), *Biochemistry 7*, 3888.

Habeeb, A. F. S. A. (1967), *J. Immunol. 99*, 1264.

Habeeb, A. F. S. A., and Atassi, M. Z. (1971), *Biochim. Biophys. Acta 236*, 131.

Heinrikson, R. L. (1970), *Biochem. Biophys. Res. Commun. 41*, 967.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Liu, T. Y., and Chang, Y. H. (1971), *J. Biol. Chem. 246*, 2842.

Lumry, R., and Eyring, H. (1954), *J. Phys. Chem. 58*, 110.

Markham, R. (1942), *Biochem. J. 36*, 790.

Moffitt, W., and Yang, J. T. (1956), *Proc. Nat. Acad. Sci. U. S. 42*, 596.

Redfield, R. R., and Anfinsen, C. B. (1956), *J. Biol. Chem. 221*, 385.

Ryle, A. P., Sanger, F., Smith, L. F., and Kitai, R. (1955), *Biochem. J. 60*, 541.

Sanger, F. (1949), *Biochem. J. 44*, 126.

Sela, M., White, F. H., Jr., and Anfinsen, C. B. (1957), *Science 125*, 691.

Shinka, S., Imanishi, M., Miyagawa, N., Amano, T., Inouye, M., and Tsugita, A. (1967), *Biken J. 10*, 89.

Singhal, R. P., and Atassi, M. Z. (1970), *Biochemistry 9*, 4252.

White, F. H., Jr. (1960), *J. Biol. Chem. 235*, 383.

White, F. H., Jr., and Anfinsen, C. B. (1959), *Ann. N. Y. Acad. Sci. 81*, 515.

Young, J. D., and Leung, C. Y. (1970), *Biochemistry 9*, 2755.