anhydride in 10 ml. of acetic acid and 5 ml. of water on the steam-bath for 20 minutes. The products (64–70% yields) were isolated as before.

The oxidation products did not depress the m.p. of authentic *p*-methoxybenzophenone and had the same infrared spectra. In each experiment, the carbon dioxide formed in the oxidations was swept with nitrogen into carbonate-free sodium hydroxide solution and converted to barium carbonate. Although the barium carbonate samples were radioactive, the yields were substantially higher than theoretical and indicated that some over-oxidation occurred. Consequently, the activities of the barium carbonate samples were not useful in determining the ratio of phenyl to *p*-methoxyphenyl migration in the rearrangement. The activities of the samples of III, the methoxybenzilic acids and *p*-methoxybenzophenones are given in Table II.

Summary

The aluminum chloride, cupric acetate and sodium hydroxide induced decarbonylations of diphenyl triketone have been shown by C^{14}-tracer studies to result with loss of the center carbonyl group.

The cupric acetate-induced decarbonylation of *p*-methoxyphenyl phenyl triketone was found to proceed in a similar manner.

The benzilic acid-type rearrangement of *p*-methoxybenzil has been found to involve preferential migration of the phenyl group.

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[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

The Molecular Weight of Lysozyme after Reduction and Alkylation of the Disulfide Bonds

BY H. FRAENKEL-CONRAT, A. MOHAMMAD, E. D. DUCAY AND D. K. MECHAM

Most proteins containing disulfide bonds are rendered insoluble when treated with mercaptans. This phenomenon is probably correlated with the known tendency of reduced proteins to form molecular aggregates, possibly through new hydrogen bonds involving their –SH groups.[2] This difficulty has at times been circumvented by performing the reduction in the presence of denaturants which serve the dual purpose of rendering the disulfide bonds more reactive, and keeping the reduced protein in solution.[3,4] There remains the further difficulty that protein –SH groups are often readily autoxidizable. Unless extensive precautions are observed, a reduced protein cannot be assumed to contain the same number of –SH groups, after it is isolated and freed from reagent mercaptan, as it had in the reaction mixture. This difficulty has been removed in the present study by alkylation of the –SH groups prior to isolation of the protein.

When lysozyme is treated with thioglycol (mercaptoethanol) in saturated urea solution at *p*H 5, it remains dissolved and its activity disappears rapidly, quite in contrast to the slow insolubilization and inactivation occurring in the absence of urea. Subsequent addition of iodoacetamide to the reaction mixture, followed by sodium hydroxide until a constant *p*H of 8.5–9 is obtained, causes alkylation of the thio groups of both the excess thioglycol and the reduced protein and thus terminates the reaction and stabilizes the product. Further, this technique facilitates the analytical evaluation of the extent of reduction, since one amide group is stably introduced into the protein for each –SH group alkylated. The loss of cystine + cysteine is an additional criterion of the extent of reduction–alkylation.[5]

In the specific case of lysozyme the alkylated product differs from the unreacted protein in its low solubility in water above *p*H 7. This affords a simple technique of separation. Depending upon the concentration of urea, and on the reaction time, more or less of the insoluble inactive product is obtained, while the residual amount of protein appears to be only slightly affected in composition as well as in enzymatic activity. Thus the reduction of lysozyme in urea, in contrast to most protein modifications, is largely an all-or-none phenomenon.

While the *amount* of insoluble inactive product formed in saturated urea depends upon reaction time, thioglycol concentration, etc., the *extent* of its reduction appears almost the same in all samples (Table I). The amount of amide nitrogen introduced is approximately equivalent to the cystine originally present, and less than 5% of the original cystine remains. In contrast, the soluble fraction, which after 20–30 minute reaction periods comprises about 50%, retains most of the original activity and shows amide-N and residual cystine values indicating that no more than one-fifth of the –S–S– bonds had been reduced and alkylated. Thus reduction of the first or second disulfide bond appears to be the rate-limiting reaction, followed by a rapid reductive cleavage of all other disulfide bonds.

Disulfide bonds have been shown to supply the crosslinks between peptide chains of proteins. Thus the sub-unit of insulin (mol. wt. 12,000) appears to consist of four peptide chains held together by 6 disulfide bonds. If lysozyme were similarly constituted, reduction–alkylation should yield a mixture of the individual peptide chains. On the basis of microbiological and chemical analyses it was recently concluded that lysozyme contained only one or two peptide chains.[6] The cleavage of

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture. Article not copyrighted.

(2) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 162 (1947).

(3) H. Fraenkel-Conrat, *J. Biol. Chem.*, **142**, 119 (1942).

(4) G. L. Miller and K. J. I. Andersson, *ibid.*, **144**, 465, 475 (1942).

(5) Principally the same technique may be, and has been, applied to the determination of –SH groups of proteins. In the absence of small-molecular mercaptans, the alkylation of protein –SH groups can be determined potentiometrically by adjusting both the protein-urea and the iodoacetamide solutions to *p*H 8.5, then mixing them and titrating the HI liberated until the solution shows a constant *p*H of 8.5 for at least 30 minutes. The product can then be isolated by dialysis and the titration results confirmed by amide-N, and possibly also cystine analyses. Egg albumin was found to contain 1.0 –SH groups per 10^4 g., in agreement with the literature.

(6) J. C. Lewis, N. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950).

TABLE I

REDUCTION OF LYSOZYME IN 8 *M* UREA FOLLOWED BY ALKYLATION WITH IODOACETAMIDE[a]

Concentration Protein	Concentration Thioglycol	Reduction time, hours	Manipulation before alkylation	Reaction product insoluble at *p*H 8.5[b] Yield, %	Amide, N, %	Extent of reaction, %[c]	Intact cystine, %[d]	Apparent mol. wt.
5.5	14	0.3	None	47[e]	13.2	93	8	13,400
		24	None	89	13.2	93		
3.8	12	2	None	84	13.2	93		15,000
		24	None	75	13.0	87		
1	20	0.3	1 Acetone pptn.	85	12.3	73		
1	20	20–24	None	87	13.0	87	4	15,000
			1 Acetone pptn.	90	13.1	90	6	22,000
			2 Acetone pptn.	85	13.4	96	9	28,000
1	33	24	1 Acetone pptn.	87	13.2	93	7	24,000
			2 Acetone pptn.	77	12.9	84	7	30,000
1	20	18–24	1 Ethanol pptn.	81	13.2	93		20,000
Untreated lysozyme, soluble at *p*H 8.5					9.2	(0)	(100)	14,400

[a] Reduction was performed in 0.3 *M* *p*H 5 acetate at 23–25°, alkylation at *p*H 7–8.5. Most experiments were performed repeatedly, and averaged. [b] This fraction showed no (*i.e.*, less than 1% of the original) activity. The soluble fraction showed amide-N, cystine, and activity values indicating that it was composed to at least 76% and in most instances to 95% of unreacted lysozyme. [c] Calculated from increase in amide-N during reaction. Assuming 8% cystine (8) to be the correct value (see text). Complete reduction–alkylation would yield an amide-N value of 13.6% of the total N. [d] Percentage of that found for untreated lysozyme (7.0%). [e] Reduction in 3 *M* or *M* urea yielded 31 and 23% of the insoluble product under these conditions.

the disulfide bonds between two similar polypeptide chains would cause a halving of the average molecular weight. However, molecular weight determinations of many preparations of reduced and alkylated lysozyme have in no case yielded a lower value than that given by the untreated protein (Table I). This finding favors the hypothesis that lysozyme is composed of a single peptide chain, internally crosslinked by disulfide bonds.[7] The molecular weight found for untreated lysozyme in 8 *M* urea solution approximates that derived from recent chemical data.[6,8,9]

While reduction-alkylation never lowers the molecular weight of lysozyme, increases, usually to about twice the original, have been observed under certain conditions. These increases can probably not be attributed to aggregation, since the osmotic-pressure runs were generally performed in 8 *M* urea. It was found that this dimerization occurs only if the reduced protein is precipitated with acetone (and to a lesser extent with ethanol) from the reaction mixture prior to treatment with iodoacetamide. A possible interpretation is that dimerization is due to formation of a disulfide bond between single particularly reactive sulfhydryl groups on pairs of reduced lysozyme molecules.

Experimental

Reduction and Alkylation.—Lysozyme was prepared by Mr. L. R. MacDonnell according to Alderton and Fevold.[10] Reaction mixtures containing 1–5% protein, 5–33% mercaptoethanol, about 0.3 *M* *p*H 5 acetate and, in most experiments 8 *M* urea were held at 23–25° for varying time periods. The mercaptan was added last, usually 1–3 hours after the urea. To terminate the reduction, the reaction mixtures were diluted with water, immediately mixed with a 10% solution of pure iodoacetamide (1 g. for 0.38 ml. thioglycol), and titrated with *N* NaOH to a stable phenolphthalein pink color (about 4.5 ml. for 0.38 ml. thioglycol). Reaction mixtures containing 20–33% thioglycol were sometimes cooled and precipitated with about 6 volumes of cold acetone or ethanol prior to treatment with iodoacetamide. Since acetone yields very bulky precipitates, these were at times redissolved and precipitated a second time. The presence of free thioglycol was evident even after two precipitations, so that autoxidation of the protein was not suspected. This technique was abandoned, however, when it was found that it led to products of markedly higher molecular weight than the original lysozyme.

Completeness of alkylation was indicated by a negative nitroprusside test, and by stability of the *p*H of the reaction mixture, even upon addition of more iodoacetamide.[5] When fractionation was not desired, the mixture was then thoroughly dialyzed (one day against running tap water at 20°, a few hours against 0.01 *N* HCl to cause solution of the protein, and then 7 days against frequent changes of distilled water at 3°) and lyophilized. More frequently the reduced and alkylated reaction product was separated by centrifugation and purified by repeated cycles of solution in 0.01 *N* HCl and precipitation at approximately *p*H 8.5, interspersed with washings with water. The soluble protein was isolated from the chilled supernatant by repeated precipitations with acetone, usually followed by dialysis. Both fractions were frozen and dried. They were then analyzed for total N by the Kjeldahl method, for amide N as previously described,[11] and at times for cystine by the Sullivan method[12] after hydrolysis with 1 ml. of 10 *N* HCl and 0.8 ml. of 88% formic acid (48 hr. refluxing). Assays were performed as previously described.[13]

The relative amounts of purified reaction product obtained under various conditions in 8 *M* urea are listed in Table I. The amide-N introduced, as calculated from the ratio of amide-N to total N in the original and modified protein, represents the most convenient measure of the extent of the reaction under various conditions. It is evident that the amount, but not the composition, of the extensively alkylated product is greatly dependent upon the reaction conditions. To calculate the extent of the reaction in terms of cystine residues affected, an accurate knowledge

(7) These results do not rigorously exclude an alternate possibility. Of two very dissimilar peptide chains, held together by one disulfide bridge, the short one might be split off and removed in the course of isolation, leaving the alkylated main chain with a similar molecular weight as the original protein. However, a few viscosity measurements (in 5 *M* urea, *p*H 7.4 phosphate) performed upon suggestion of a reviewer, gave consistently higher values for the reduced and alkylated, as compared to untreated lysozyme. Though all values were low at the concentrations used (0.8, 0.4%), this finding is in accord with the concept of a single folded peptide chain, opened and thus lengthened by rupture of the disulfide bonds.

(8) C. Fromageot and P. de Garilhe, *Biochem. Biophys. Acta*, **4**, 509 (1950).

(9) In the absence of urea, in solution buffered with phosphate to *p*H 7.4, a slightly higher value was obtained (see experimental).

(10) G. Alderton and H. L. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).

(11) H. Fraenkel-Conrat and H. S. Olcott, THIS JOURNAL, **70**, 2673 (1948).

(12) M. X. Sullivan, *U. S. P. H. Repts.*, **44**, 1600 (1929); analyses were kindly performed by Mrs. R. B. Silva.

(13) H. Fraenkel-Conrat, *Arch. Biochem.*, in press.

of the number of such residues occurring in untreated lysozyme is prerequisite. Fromageot and de Garilhe[8] found 8.0% cystine, *i.e.*, 5 cystine residues per mole, a value which together with the methionine value of 2.06%[6] accounts for the sulfur of lysozyme quantitatively. Analyses performed in connection with the present study have yielded an average value of 7.0%, but in view of the lability of the cystine of many proteins during acid hydrolysis we favor the higher value quoted above. On that basis, quantitative reduction and alkylation would yield a calculated amide-N content of 13.6% of the total N, while reaction of four of the five supposed cystine residues would give a preparation containing 12.7% (amide-N of total N). Since many samples actually showed values of 13.2–13.6%, it appears that they consisted mainly of completely reduced lysozyme. The average of all thoroughly reduced preparations, isolated in a variety of ways, showed a value of 13.1% which is equivalent to reduction of all the cystine actually found by us, and to 4.5 of the 5 residues believed[8] to occur per molecule. Cystine analyses indicate destruction of 91–96% of this amino acid. This may well represent complete absence of intact cystine, since carboxymethylcysteine, the hydrolytic product of the alkylated cysteine residues, yields a small amount of cystine (5%) during "hydrolysis" with 6 *N* HCl.[14]

Some experiments were performed to study the over-all rate of the reaction in urea by analyzing the unfractionated reaction product. As Fig. 1 shows, 13% thioglycol caused complete reduction in 45 minutes; with 33% thioglycol, the reaction was finished in 20 minutes. The reduction of lysozyme was also repeatedly performed in the absence of urea. High thioglycol and low protein concentrations (*e.g.*, 20 and 1%) prevented gelation or extensive precipitation of the product. After treatment with iodoacetamide in the usual manner, from 20–67% of the protein was insoluble after reaction periods of 2–18 hours. Only after the shortest reduction period did the insoluble product show lytic activity (40%). The water-soluble fractions isolated in 61–12% yield, appeared to represent partially reduced products, retaining 40–50% of their original disulfide bonds, and 70–85% of the original activity. The possibility that they represented heterogeneous mixtures was not excluded. In any case the reaction was much slower and less clear-cut in the absence than in the presence of urea.

Osmotic Pressure Measurements.—To prevent aggregation of the protein molecules, osmotic pressure measurements were generally performed in 8 *M* urea solution. The protein was dissolved in little water, containing a trace of HCl (about 0.01 *N*) and then treated with sufficient 10 *M* urea, 2 *M* NaCl and 3 *M* sodium acetate to give final concentration of 8, 0.1 and 0.1 *M*, respectively. Finally the *p*H was adjusted to 7.3–7.5 with *N* acid or alkali if necessary. Protein concentration was usually 0.5–0.8%. The protein-free buffer was prepared in the same manner and adjusted to the same *p*H. The technique of osmometry was the same as used in previous studies.[15]

The final protein concentration which varied by no more than 10% from the original was verified by Kjeldahl analysis after exhaustive dialysis. The apparent molecular weights were calculated using 18.6% as the N content of dry isoelectric lysozyme and 19.0% as the N content of the derivative. The results of many analyses of reduced and alkylated lysozyme preparations by this method have been averaged and summarized in Table I.

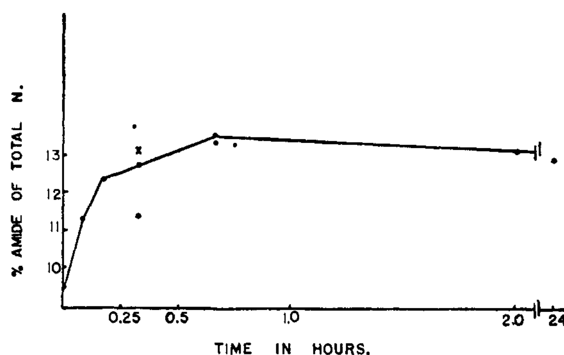

Fig. 1.—Rate of reduction of lysozyme by thioglycol in 7–8 *M* urea at *p*H 5.0: protein concentration, 3.5–3.8%; thioglycol concentration, xx, 33%; ●●, 13%; OO; 4.3%.

Untreated lysozyme was analyzed both in urea solution as described above and in phosphate buffer without urea. Early in this investigation, lysozyme was found to pass at an appreciable rate through the dialysis membranes then available, so that the maximal osmotic pressure had to be ascertained by extrapolation from the slope of the pressure decreasing with time from the 3rd to 24th hour. Values of approximately 15,000 were obtained by this technique.

With the dialysis tubing now available, only very little of the protein diffused (5%) in 8–10 days. This appeared to be lysozyme and not a contaminating peptide, since a similar amount passed through the same membrane when the same preparation was equilibrated a second time against fresh buffer. The molecular weight as calculated from these runs and after correction for the protein content outside of the membrane was 16,600. This is definitely higher than the value obtained in urea solution (Table I), suggesting that the protein is partly aggregated in 0.5% solution at *p*H 7.5.

Summary

1. The disulfide bonds of lysozyme dissolved in 8 *M* urea are readily reduced by thioglycol at *p*H 5. Reduction of each molecule, once started, appears to proceed rapidly to completion, since partially reduced reaction mixtures consist of two clearly defined fractions, one almost completely reduced and the other unreacted. In the absence of urea, the reaction is much slower and leads to partially reduced fractions.

2. The reduced protein can rapidly and quantitatively be alkylated with iodoacetamide at *p*H 7 to 8.5. The product of the reaction is insoluble at that *p*H.

3. The molecular weight of the reduced and alkylated lysozyme is the same as that of the original protein. This suggests that lysozyme consists of a single peptide chain, internally crosslinked by disulfide bonds.

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(14) When it was attempted to use insulin as a means of testing the technique, this protein was found very much more resistant to extensive reduction by thioglycol at *p*H 5 and 7.5, with or without urea.

(15) H. Fraenkel-Conrat and D. K. Mecham, *J. Biol. Chem.*, **177**, 477 (1948).