Formation of Three-Dimensional Structure in Proteins. I. Rapid Nonenzymic Reactivation of Reduced Lysozyme*

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ABSTRACT: Rapid regeneration of enzymic activity from reduced, inactive chicken egg-white lysozyme has been achieved with mixtures of oxidized and reduced thiols. The maximal rates obtained require less than 5 min for 50% regeneration. Regeneration periods of 30 min routinely yield 60–85% of the native lysozyme activity. Regeneration studies at several ratios and absolute concentrations of reduced and oxidized glutathione (GSH and GSSG) show maximal rates at (GSSG)/(GSH) = $^1/_{10}$ and when (GSH) = 5×10^{-3} M. Regeneration systems containing cystine and cysteine, or cystamine and cysteamine, resulted in rates and yields similar to those obtained with glutathione at the same levels of disulfide and thiol. Ion-exchange chromatography showed the major product of rapid regeneration to be the same as native lysozyme. This major fraction of regenerated lysozyme also had the same specific activity and circular dichroic spectra as native lysozyme. Air reoxidation and regeneration is strongly inhibited by EDTA, while the optimal glutathione system is unaffected by EDTA. The kinetic consequences of varying temperature, pH, and preincubation of reduced lysozyme in various denaturing solvents have been explored. Circular dichroic studies show that a very rapid chain folding is completed before protein disulfides are formed. Moreover, in the optimal range of conditions for rapid regeneration of activity, the regeneration rate is independent of thiolate ion concentration. Therefore neither major chain folding nor the covalent chemistry of thiol–disulfide exchange appears to be rate limiting. We infer that what is rate limiting in the reactivation process is the conformational changes that accompany protein thiol–disulfide interchange reactions. Recently published values of GSSG and GSH levels in mammalian tissues correspond closely to the glutathione levels in the rapid regeneration system reported here. Thus our *in vitro* rapid regeneration system appears to be a highly plausible model for the biosynthetic formation of three-dimensional structure of lysozyme.

In the terminal stages of protein biosynthesis partly or fully assembled polypeptide chain undergoes a transition from one-dimensional to three-dimensional structure that is finally functionally active. In the case of disulfide containing proteins this process also involves coupling of sulfhydryl groups. The possibility exists that these chain-folding and disulfide-forming processes could be rate limiting in the overall production of a functionally active protein molecule, and therefore deserves to be further investigated. *In vitro* regeneration of native proteins from disulfide-cleaved proteins are convenient models for this purpose and a recent summary of such experiments has been presented by Anfinsen (1967). Substantial experimental support has been developed for the idea introduced by Sela *et al.* (1957) that the amino acid sequence of a protein is sufficient to determine its three-dimensional structure. However, the *in vitro* regeneration rates are generally much smaller than the apparent overall rates of protein biosynthesis, which range from several seconds to a few minutes (Bishop *et al.*, 1960; Dintzis, 1961; Canfield and Anfinsen, 1963; LaCroute and Stent, 1968; Wilhelm and Haselkorn, 1970). Experiments measuring rates of protein biosynthesis, to be sure, have not yet revealed how fast the biologically active structures are formed on or off the ribosomes. In such experiments a time lag of at least several minutes occurs between stopping amino acid incorporation and measurement of biological activity. It is obviously possible and even likely that some folding of the nascent polypeptide chains occurs during this interval. Even so, the experiments indicate that chainfolding takes no more than a few minutes. The attractiveness of the model would increase if the *in vitro* rates of regeneration could be made to approach the biological range.

The times for half-regeneration in the air oxidation of lysozyme were 15–20 min in the most rapid cases (Epstein and Goldberger, 1963; Yutani *et al.*, 1968); the air oxidation of ribonuclease is considerably slower (White, 1967, and references therein). The mechanism of refolding of reduced lysozyme in air oxidation medium has been investigated by Isemura's group (see Yutani *et al.*, 1968). Upon dilution of a solution of reduced lysozyme in 8 M urea into a Tris-Cl buffer of pH 8, they observed by several optical criteria, "instantaneous reappearance of 70% of native α helix." This occurred before any oxidation of sulfhydryls occurred. Upon reoxidation they were able to recover full structural identity and enzymic activity. The rate of regeneration was a function of copper ion concentration; the fastest yield reported was 40% in 20 min.

The regeneration of lysozyme and several other reduced proteins is accelerated by a microsomal enzyme preparation discovered by the groups of Anfinsen (Goldberger *et al.*,

* From the Department of Biochemistry, College of Medical Sciences, University of Minnesota, Minneapolis, Minnesota 55455. *Received July 16, 1970.* This research was supported by U. S. Public Health Service Grant GM 10900 and a grant from the Cancer Coordinating Committee of the University of Minnesota (V. S.) for technical assistance. A preliminary account of some of these results has already been published (Wetlaufer and Saxena, 1968).

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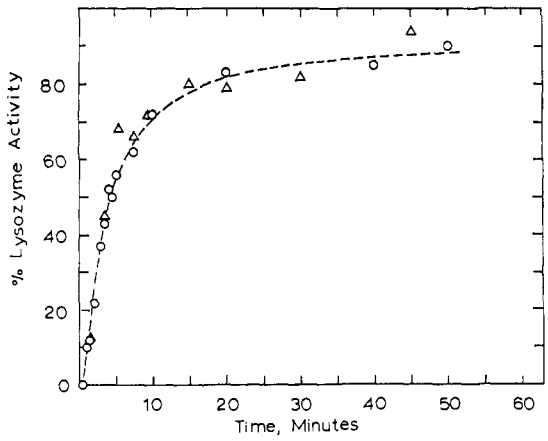


FIGURE 1: Equivalence of two assay procedures for lysozyme activity. Assay immediately after aliquoting, O; assay after arresting reactivation, Δ, as detailed in the Experimental Section. Temperature, 37°; pH 7.85; buffer 0.08 M Tris; lysozyme, 1.0×10^{-6} M; GSH, 3.0×10^{-3} M; GSSG, 3.0×10^{-4} M.

1963), and of Straub (Venetianer and Straub, 1963). This enzyme is apparently present in several tissues; it has been characterized as to mode of action (Venetianer and Straub, 1964; Steiner *et al.*, 1965), and has been highly purified and characterized as to composition, molecular weight, and properties (DeLorenzo *et al.*, 1966). By addition of this enzyme to the reactivation medium Goldberger *et al.* (1964) were able to reactivate reduced lysozyme and reduced ribonuclease to half of their theoretical activity in about 5 min. Soybean trypsin inhibitor (Steiner *et al.*, 1965) reactivation was also accelerated by this enzyme. It is claimed that the function of the microsomal enzyme is to reshuffle quickly the unnatural disulfide bonds that may be formed in the initial stages of the refolding process (Givol *et al.*, 1964).

A thorough study of the regeneration of enzymic activity from the mixed disulfide of lysozyme and cystine has been carried out by Bradshaw *et al.* (1967). This mixed disulfide could be rapidly reactivated (with a half-regeneration time of about 5 min) by addition of mercaptoethanol or other low molecular weight thiol. These investigators showed that the reactivation proceeded by a disulfide rearrangement mechanism.

We have been exploring additional possibilities of accelerating renaturation rates of a number of disulfide-reduced proteins. In the present studies, we show that during reactivation of reduced chicken egg-white lysozyme oxidation and disulfide rearrangement can be carried out simultaneously with regeneration rates at least as high as those obtained by Bradshaw *et al.* (1967) for the process involving disulfide rearrangement of preoxidized lysozyme and comparable to those obtained by Goldberger *et al.* (1964) for the process assisted by the disulfide-shuffling microsomal enzyme preparation. The essential feature of the present regeneration system is use of a mixture of reduced and oxidized low molecular weight thiol. Systematic studies of the rate of regeneration of reduced lysozyme as a function of the ratios and concentrations of thiol and disulfide are presented. Several low molecular weight thiol, disulfide pairs are compared and all found to have about the same efficiency, both in rate and in final yield. It is found that the fastest regeneration occurs with thiol–disulfide levels that correspond fairly well with the known physiological levels.

Experimental Section

Materials. Twice-crystallized chicken egg-white lysozyme was obtained from Worthington Biochemical Corp. (lot Ly7GA, minimum activity, 5000 units/mg). "Tris" was obtained as the free base, Trizma quality, from Sigma Chemical Co. as were reduced glutathione and oxidized glutathione. Analysis by Ellman's method (Ellman, 1959) showed the SH content of these compounds to be 99 and 0.4% of stoichiometry, respectively. Urea and EDTA were AR grade products of Mallinckrodt Chemical Works. Mercaptoethanol was obtained from Eastman Organic Chemicals and was redistilled before use. Gu·HCl[1] was spectrophotometric grade 6.0 M solution supplied by Heico Inc. Dried cells of *Micrococcus lysodeikticus* were obtained from Mann Research Laboratories. Other reagents were of Reagent grade. Water was deionized, followed by glass distillation from 0.2 N H_2SO_4.

Lysozyme Assay. A modification of the method of Jolles (1962) was used. Standard solutions of native lysozyme were prepared at several concentrations between 0.50 and 2.50 mg per 100 ml in a buffer solution whose composition corresponded to that of a subsequent reactivation experiment. *M. lysodeikticus* dried cells were prepared in suspension at a concentration of about 25 mg/100 ml in 0.067 M phosphate buffer, containing 0.10% NaCl, of pH 6.2. To 5.00 ml of the cell suspension, 1.00 ml of protein solution was added, mixed well, and transferred to a 1.00-cm path-length cuvet. The decrease in apparent absorbance at 650 nm was measured in a Zeiss PMQII spectrophotometer up to 90 sec from the instant of mixing enzyme with cell suspension. When the differences in apparent absorbance at 30 and 90 sec are plotted *vs.* enzyme concentration, a straight line passing through the origin is obtained.

Another modification of the assay method was used to obtain more data points during the first few minutes of a rapid reactivation process. This consisted of rapidly acidifying an 1.00-ml aliquot of enzyme solution from the reactivation solution by rapidly adding it to 1.00 ml of 0.10 M acetic acid (final pH 5). This effectively arrests further reactivation for at least 30 min as judged by constancy of lytic activity. In this modified assay procedure twice the usual volume of cell suspension was used. The results of the two-assay procedures are consistent, as is shown in Figure 1. Standardizing assays were carried out within 1 hr or 2 hr before every regeneration experiment to ensure identical conditions in the standard and test systems. Neither glutathione nor EDTA showed any effect on the lysozyme assay at concentrations obtained from aliquots of the regeneration solutions.

Preparation of Reduced Lysozyme. We followed the procedure of Anfinsen and Haber (1961) in using 1.0 ml of mercaptoethanol/mg of protein in 8 M urea at pH 8.5. Details were similar except that we used Tris in place of methylamine for buffering. After reduction, the solutions were acidified to pH 3 with acetic acid, and passed through a Sephadex G-25 column equilibrated and eluted with 0.1 M acetic acid. After separation of the reduced lysozyme from urea and excess

[1] Gu·HCl, guanidinium hydrochloride.

mercaptoethanol, the protein was precipitated by addition of five volumes of a 39:1, v/v mixture of acetone–1 N HCl at 0°. The protein was collected by centrifugation, washed with dry ether, and dried under vacuum. Alternatively the reduced lysozyme solution in 0.1 M acetic acid was frozen and lyophilized. In either case the dried reduced protein was stored in a closed container in a desiccator charged with anhydrous $CaSO_4$ at 2°. This resulted in a preparation whose SH content was 7.6–7.8 equiv/mole. It was completely inactive enzymically. When stored as indicated, there was no oxidation of protein thiols over a period of several months. In contrast, reduced lysozyme in 0.1 M acetic acid at 2° showed significant loss of SH titer after 2 days. In all studies reported below either lyophilized material or the dried powder was used.

Reactivation Experiments. In all experiments the concentration of reduced lysozyme was 1.0×10^{-6} M ± 10%. At higher concentrations of reduced protein we found that regeneration results in the formation of partly insoluble products, in agreement with the experience of Isemura *et al.* (1961) and White (1967). Lysozyme concentrations were determined photometrically, using $A_{280}^{1\%}$ 26.3 for the native protein (Sophianopoulos, 1962; L. Clauss, 1969, personal communication), and $A_{280}^{1\%}$ 23.7 for the reduced protein in 0.1 M acetic acid (L. Clauss, 1969, personal communication).

Reactivation media containing desired amounts of GSH, GSSG, NaCl, Tris, acetic acid, and in some cases EDTA were prepared 1 or 2 min before their use as a precaution to prevent appreciable air oxidation of GSH. To 9.0 ml of this medium was added 1.00 ml of 10^{-5} M reduced lysozyme, prepared in 0.1 M acetic acid to start the reactivation process. Constant ionic strength of 0.10 was maintained in the final mixture. The pH of the final mixture was determined at the temperature of each regeneration experiment.

Ion-Exchange Chromatography. Commercial preparations of hen egg-white lysozyme have been demonstrated to be inhomogeneous by ion-exchange chromatography by Stevens and Bergstrom (1967). Using their procedure we observed a similar inhomogeneity in the Worthington lysozyme used in the present studies. The protein was resolved into several peaks (Figure 2) under experimental conditions described in legend for Figure 2. A few test experiments showed however that, after disulfide cleavage, peak A, peak B, and unfractionated lysozyme were reactivated in identical media at identical rates. Reactivated peak A lysozyme, after glutathione removal, concentration and lyophilization, was compared with peak A native lysozyme on an analytical ionexchange column described in legend for Figure 8. In a control experiment native lysozyme (peak A, Figure 2) was added to a reactivation medium containing GSH and GSSG and subjected to the same concentration, isolation and lyophilization procedures as the reduced peak A lysozyme. This material showed the same behavior on the analytical column as did peak A material applied directly to the column. Diaflo (PM-10) filtration under N_2 pressure of 25 psi and Sephadex G-25 chromatography were used in concentration and isolation procedures.

Circular Dichroism. Measurements were performed with a Jasco Durrum Model CD-SP J-10 instrument. Protein concentration was 10^{-4} M and sample path length was 0.10 mm. With a circular dichroic sensitivity scale of 3.19×10^{-5} deg/cm, spectra were taken between 250 and 210 nm. In some experiments when protein concentration of 10^{-6} M and sample path length of 1 cm was used, the same CD spectra were observed. A mean residue weight of 115 was used in calculation of ellipticities. All experiments were performed at 25 ± 0.5°.

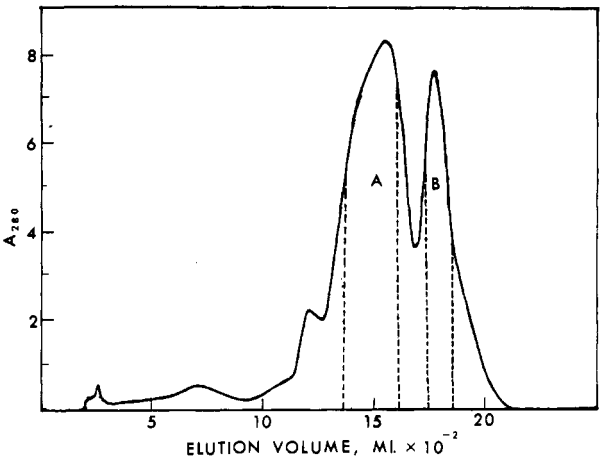


FIGURE 2: Preparative Bio-Rex 70 chromatography of Worthington 7GA lysozyme on a 75 × 3 cm column equilibrated and eluted with 0.2 M phosphate buffer of pH 7.14 at 25°. Lysozyme loaded on column: 1.8 g in 19 ml of the buffer. Flow rate: 1 ml/min. Fractions were collected at 10-ml intervals and read on Zeiss II spectrophotometer for absorbance at 280 nm. Fractions between dotted lines under peaks A and B are referred to as peak A and peak B materials.

Results

Figure 1 shows typical results for a rapid reactivation of reduced, inactive lysozyme. The data points depicted here refer to two regeneration experiments, performed one immediately after the other, under identical conditions but using in one case the conventional assay, and in the second case the rapid assay procedure for enzymic activity. Equivalence of the two assays is apparent. A lag period of 0.5 to 1 min is characteristic of these regenerations. In Figure 1 and in all subsequent figures and discussion, "per cent lysozyme activity" represents per cent of the specific activity of native lysozyme; in other words, the per cent of the total activity expected on the basis of the amount of protein used.

Effect of Thiol and Disulfide Concentration. The effect of systematic variation in GSH and GSSG levels upon reactivation is shown in Figures 3 and 4. The percentage regain of activity after 5 min is shown in Figure 3 by the extent to which the circles are blackened. The corresponding data for regain of activity in a 30-min period appears in Figure 4. Figure 3 shows the pattern of the dependence of regeneration rate on GSH and GSSG concentration; Figure 4 shows the corresponding pattern of yield on the same variables. It can be seen that those GSH and GSSG concentrations resulting in rapid regenerations also result in high yields. We call special attention to the finding that in the higher range of GSH concentrations, the increase in 5-min yields is but slight (about 54% to about 60%) when thiol concentration is increased *tenfold* at constant GSH/GSSG. Specifically we are comparing 8×10^{-4} M GSH, 4×10^{-5} M GSSG (5-min activity = 54%) with 8×10^{-3} M GSH, 4×10^{-4} M GSSG

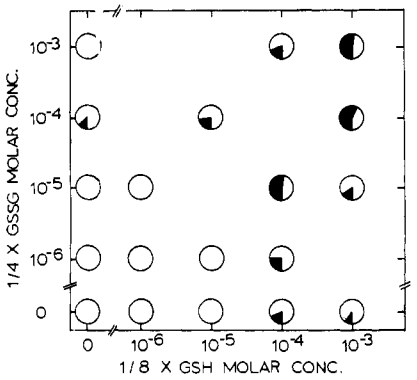


FIGURE 3: Effects of differing initial concentrations and ratios of oxidized glutathione (GSSG) and reduced glutathione (GSH) on the rate of regeneration of reduced hen egg-white lysozyme. The extent of recovery of lysozyme activity in 5 min is shown by the extent of circle darkening (50% recovery of the specific activity of native lysozyme would be represented by a half-darkened circle). The regeneration conditions were 37°, pH 8.0, 0.08 M Tris buffer, initial lysozyme concentration, 10^{-6} M. Enzymic acitivity was measured on aliquots quenched to pH 5.0.

(5-min activity = 60%). The concentration range providing most rapid regeneration was 3–6 × 10^{-3} M GSH, and 3–6 × 10^{-4} M GSSG, but tenfold dilution of both GSH and GSSG decreases the rate by only 10%.

At selected concentrations in the range of 8 × 10^{-4} to 8 × 10^{-3} M for thiol and 4 × 10^{-5} to 10^{-4} M for disulfide compound, efficiency of regeneration was found within experimental error (±5%) to be the same for cysteine–cystine, cysteamine–cystamine, and GSH–GSSG pairs.

Effect of Temperature. Both in initial rate and in final yield (roughly represented by the 5- and 30-min yields, respectively), reactivation is slightly more rapid and more extensive at 37° than at the higher or lower temperatures.

Effect of pH. In a series of regenerations the rate of regeneration was found to be invariant with pH over the range 7.4–8.2. This range of pH independence was found to hold at three temperatures: 32, 37, and 42°. These results are summarized in the form of 5- and 30-min yields of active enzyme, in Table I. In all these experiments GSH and GSSG were 6 × 10^{-3} and 6 × 10^{-4} M, respectively.

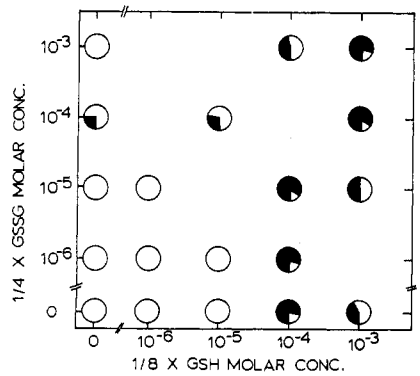


FIGURE 4: Effects of differing initial concentrations and ratios of GSSG and GSH on the extent of recovery of lysozyme activity after 30 min. Details are the same as for Figure 3.

TABLE I: Effect of pH and Temperature on Lysozyme Reactivation in Presence of GSSG and GSH.

Temp (°C)	pH	5-min Yield (%)	30-min Yield (%)
32	7.5	43	57
	8.2	43	62
37	7.4	54	82
	7.85	54	85
	8.0	51	73
	8.2	56	75
42	6.9	18	44
	7.5	40	64
	8.0	40	66

Effect of EDTA. Early in our work we routinely carried out control regenerations omitting addition of low molecular weight thiol and disulfide. We found that these so-called air control regeneration experiments show more variability in rates than any other regeneration system. Yutani *et al.* (1968) have shown that regeneration rates differ with different concentrations of copper ion in the air oxidation system. On the hypothesis that variable traces of metal ions are responsible for variability in the air control, regeneration experiments were carried out in the presence of 10^{-4} M EDTA. No significant regeneration was seen in air controls containing EDTA in several experiments, over a period of 30 min.

EDTA shows no significant effect on the efficiency of most rapid glutathione regeneration systems as typified by data in Figure 5. However, at lower GSH or GSSG concentrations EDTA did show an effect. The most marked effect was seen in the case when GSH was 8 × 10^{-3} M and GSSG was not introduced into the reactivation medium. Without EDTA, 20% of activity was regained in 5 min whereas with 10^{-4} M EDTA no measurable activity appeared in the same length of time.

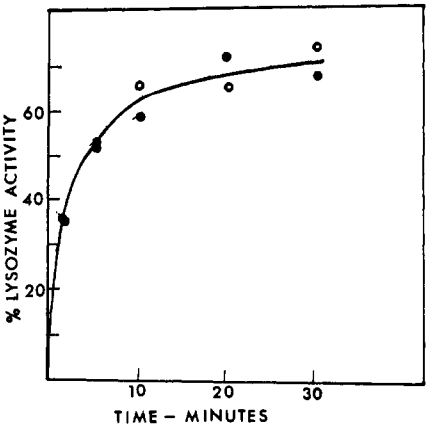


FIGURE 5: Effect of EDTA on lysozyme regeneration system containing 6 × 10^{-3} M GSH–6 × 10^{-4} M GSSG. Temperature 37°, pH 8, 0.1 M Tris-acetate buffer. Open points: experiments with 10^{-4} M EDTA; filled points: experiments without EDTA.

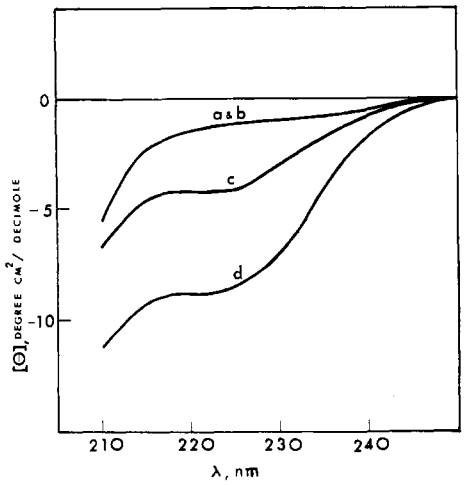


FIGURE 6: Circular dichroic spectra of reduced lysozyme in various solvents: (a) in 6 M Gu·HCl, (b) in 8 M urea, and (c) in 0.1 M acetic acid. Circular dichroic spectrum of native lysozyme; (d) in 0.1 M Tris-acetate buffer (pH 8).

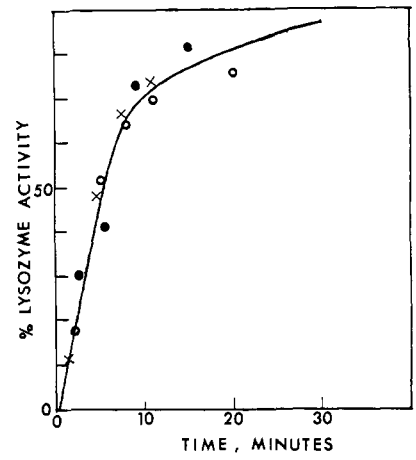


FIGURE 7: Reactivation of reduced lysozyme upon 100-fold dilution from three different solutions into optimum reactivation medium: (○) 6 M Gu·HCl, (●) 8 M urea, (×) 0.1 M acetic acid. Composition of each reactivation medium: protein, 1.0×10^{-6} M; GSH, 5×10^{-3} M; GSSG, 5×10^{-4} M; buffer, 0.1 M Tris-acetate (pH 7.85) 37°.

Effect of Preincubation. Before reactivation, reduced lysozyme was dissolved and allowed to stand for 15–30 min in three different solvents, *viz.*, 6 M Gu·HCl, 8 M urea, and 0.1 M acetic acid. No oxidation of protein sulfhydryls occurred during this period. The protein concentration of 10^{-4} M, urea, and Gu·HCl solutions of lysozyme displayed a circular dichroic spectrum in the 250 to 210 nm range markedly different from that of native lysozyme (see Figure 6). In 0.1 M acetic acid the spectrum retains features qualitatively similar to that of the native enzyme. Regeneration experiments started by 100-fold dilution of Gu·HCl, urea, and acetic acid solutions of reduced lysozyme, however, produced within experimental error identical regeneration kinetics (Figure 7).

Characterization of Glutathione-Regenerated Lysozyme. The isolated (peak A, Figure 2) reduced-reoxidized lysozyme showed less than 0.1 free SH equiv/mole. Upon ion-exchange chromatography the reduced-reoxidized material showed a clear separation into three peaks (peaks a, b, and c of Figure 8). Experimental details are given in the legend for that figure. These three fractions constituted, respectively, 20, 13, and 67% of total protein. With respect to enzymic activity, A was inactive, B was 53% active, and C was 100% active compared to activity of peak A (Figure 2) lysozyme. The major fraction C of reduced-reoxidized lysozyme coincided in position of elution with rechromatographed native lysozyme (curve d, Figure 8) from peak A (Figure 2). Fraction C also showed circular dichroic identity with native lysozyme in 250- to 210-nm wavelength region.

Discussion

Application of the column chromatographic identity test to lysozyme regenerated under rapid regeneration conditions with glutathione shows (Figure 8) three main components. The most abundant of these (peak c) is identical with the purified fraction used as the starting material chromatographically, in its circular dichroic spectrum, and in specific activity. The less abundant peak b material also shows enzymic activity, but a specific activity significantly lower than the starting material. The formation of two different active lysozyme species on regeneration was also observed by Bradshaw *et al.* (1967); these earlier workers also observed a very small amount of a third active fraction. Although the chromatographic systems of Bradshaw *et al.* are not identical with ours, they are close enough that it is probable that their second active lysozyme is the same as our peak b, Figure 8 material. We have no additional suggestions to add to those of Bradshaw *et al.* as to the possible origins of chromatographically different lysozyme components. While the presence of minor components in regenerated lysozyme is not understood, this cannot obscure the finding that the major

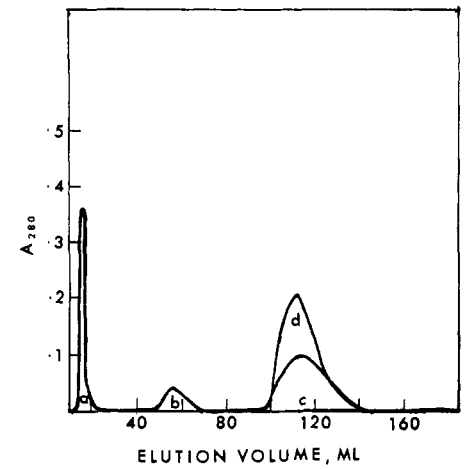
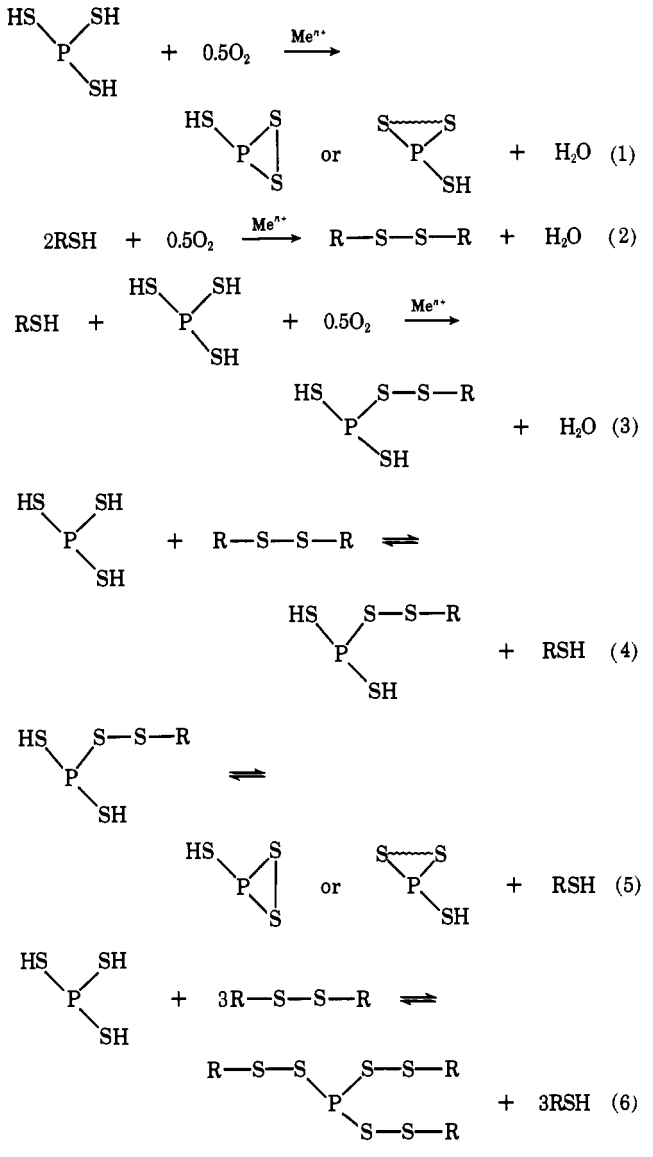


FIGURE 8: Analytical Bio-Rex-70 chromatography: d, native lysozyme of peak A of Figure 2; reduced glutathione-reactivated lysozyme, a, b, and c. Column dimensions 25 × 1 cm. Column equilibrated and eluted with 0.2 M phosphate buffer (pH 7.1) at 25° at a flow rate of 24 ml/hr. Amount of lysozyme applied: 0.5 ml of buffer solution containing 1 mg of protein. Absorbance at 280 nm was measured continuously on automatic ultraviolet analyzer.

SCHEME I



component of rapid regeneration is identical with the initial lysozyme.

The transformation of a disulfide-cleaved disorganized protein to a reoxidized functionally active structure can be conveniently viewed as comprised of three kinds of rate processes: (a) polypeptide backbone refolding: change from a disorganized three-dimensional structure to a structure at least partially resembling that of the native protein; (b) formation of intramolecular disulfides: either native or mismatched; and (c) shuffling mismatched disulfide bonds to form native disulfide bonds. The task of analyzing the kinetics of regain of lysozyme activity into absolute contributions from the three-component processes is difficult because of the processes' probable interdependence. However, we have evidence to show that process a and covalent chemistry of b and c are not rate determining in our experiments where the regeneration of lysozyme activity occurs most rapidly.

A. POLYPEPTIDE BACKBONE FOLDING. When reduced lysozyme in 8 M urea is strongly diluted with aqueous buffer "instantaneous" recovery of substantial circular dichroic characteristics of native lysozyme takes place before reoxidation of sulfhydryls occurs (Yutani *et al.*, 1968).[2] The rapidity of this process is remarkable in comparison to rate expectations based on a random search mechanism (Levinthal, 1968, 1969). In our reactivation media, because of the presence of GSH and GSSG, it is not possible to sort out folding from formation and shuffling of disulfide bonds. We have approached this difficulty by carrying out preincubation-regeneration experiments. At the preincubation stage we have put reduced lysozyme in different structural states, as reflected by the circular dichroic curves a, b, and c of Figure 6. It is readily seen that whereas reduced lysozyme in 6 M Gu·HCl or 8 M urea has little or no circular dichroic similarity to native lysozyme (curve d) in 0.1 M acetic acid there is considerable qualitative resemblance to the native protein. In Gu·HCl and urea the spectrum resembles that of several other denatured proteins (Dearborn and Wetlaufer, 1970). A uniform random state in this solvent would be expected on the basis of Tanford's (Tanford *et al.*, 1967) experiments with Gu·HCl as protein denaturant. Yet when these different solutions are diluted identically with an alkaline buffer containing GSH and GSSG, the regeneration kinetics are identical (Figure 7). It follows that the rate of major polypeptide backbone refolding is relatively rapid and that the same state of folding is reached from reduced lysozyme in different initial states.

B. FORMATION OF INTRAMOLECULAR DISULFIDE CROSS-LINKS. There are two recognized routes (relevant to this work) by which protein sulfhydryls may be oxidized to form disulfide bonds. These are, air oxidation catalyzed by trace metals (Cecil and McPhee, 1959; Cecil, 1963; Ahmed, 1969), and sulfhydryl–disulfide exchange (Lamfrom and Nielsen, 1957; Cecil and McPhee, 1959; Cecil, 1963; Jocelyn, 1967). These processes require the ionized thiolate species. However, to simplify we do not write the anionic forms, and show only three protein SH groups in Scheme I. In a protein molecule, P, a native disulfide bond is implied by a straight line between two S atoms, while a mismatch is implied by a zigzag line between S atoms.

The catalytic role of metal ions is described by the first three reactions. If no additional thiols or disulfide compounds are introduced in the reoxidation medium, reaction 1 should represent the only oxidative mechanism. Accordingly when metal ions are also precluded from participation, for example, by addition of EDTA, we observe no significant reoxidation or regeneration over a 30-min period. Over longer periods of time, it is possible that air oxidation of thiols may occur without metal ion catalysis. The variability of air-oxidation (control) experiments is in all likelihood due to variations in the trace concentrations of catalytically active metal ions (Ahmed, 1969).

Thiols have been shown to assist in the renaturation of reduced lysozyme (Epstein and Goldberger, 1963). Since

[2] Yutani *et al.*'s interpretation of these results, while attractive, is not unique. It is just as possible that arrangements of the lysozyme polypeptide chain containing none of the structural (folding) components of native lysozyme could give rise to the circular dichroism spectrum seen after rapid dilution of reduced lysozyme. Or the observed circular dichroism spectrum could be the result of a distribution of rather dissimilar structures over the whole population of peptide chains. These questions are currently under study in our laboratory.

thiols cannot *per se* serve as oxidant, oxidation is presumably carried out by reactions 2 and 3 in addition to reaction 1. As an illustration, in a regeneration experiment when GSH was 8×10^{-3} M and no GSSG was introduced into the reactivation medium, 20% of lysozyme activity was regained in 5 min. With 10^{-4} M EDTA added to an otherwise identical regeneration, no measureable activity appeared in the same length of time because air oxidation of the thiols present was greatly retarded. Reactions 1–6 may all occur when both RSH and RSSR are initially introduced in the reoxidation medium. But in our most rapid regeneration systems, addition of EDTA had no effect on the regeneration kinetics. We may conclude that reoxidation in this system occurs entirely *via* sulfhydryl–disulfide exchanges.

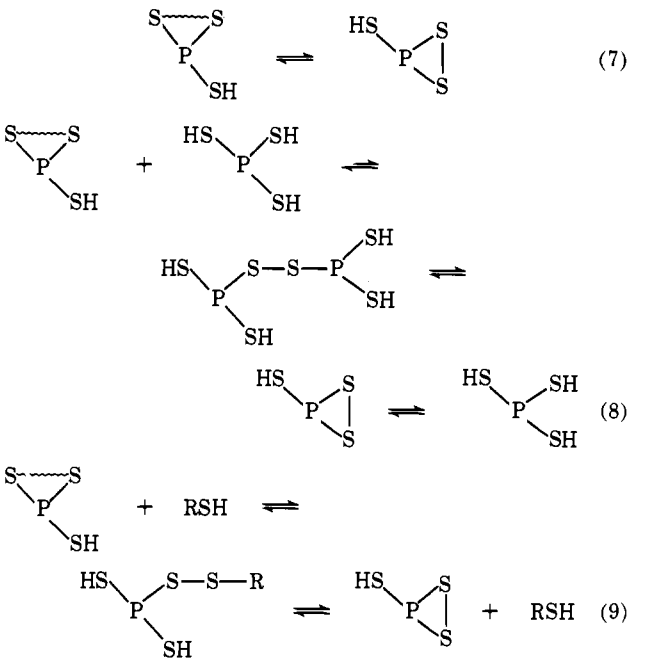
Reaction 6 shows overoxidation of protein sulfhydryls with high concentrations of RSSR. If all eight SH groups of reduced lysozyme are exchanged with low molecular weight disulfides an inactive species is formed (Bradshaw *et al.*, 1967).

We do not yet have data on the rates of reoxidation of lysozyme sulfhydryls in the rapid glutathione regeneration system. Recall, however, that regeneration rates increase only slightly (53–60% at 5 min) when GSH is increased fourfold (8×10^{-4} to 30×10^{-4} M) (maintaining constant GSH/GSSG, and not at all with a further increase (30×10^{-4} to 80×10^{-4} M). These findings strongly show that thiol, disulfide interchange is not rate limiting in the overall process. Essentially the same inference may be drawn from the pH insensitivity of regeneration kinetics in the pH range 7.4–8.2, since this range lies in the range of pK of thiols (Cecil, 1963). Rate studies on model thiol–disulfide-exchange reactions (Eldjarn and Pihl, 1957; Lamfrom and Nielsen, 1958; Jocelyn, 1967) confirm the thiolate anion as the obligate reactive species. These investigators report second-order rate constants of the order of 10^5 l. min^{-1} mole^{-1} for exchange reactions of the type $^1RSS^2R + {}^3RS^- \rightarrow {}^1RSS^3R + {}^2RS^-$. While these model compound studies deal with intermolecular exchanges, exchanges in the regeneration systems are both inter- and intramolecular. A better system for rate comparison might be the reversal of reaction 6. This reaction as studied by Bradshaw *et al.* (1967) removes four of the eight (extra) half-cystine residues in 15 sec under conditions comparable to those of our rapid regenerations.

C. Shuffling of mismatched disulfide bonds. There are several possibilities of shuffling of protein disulfide bonds all involving sulfhydryl–disulfide interchange. Thus such interchanges may occur on the same protein molecule, between two protein molecules, or between a protein molecule and a low molecular weight thiol. These processes are schematically represented by Scheme II. In air reactivation of reduced proteins, correction of wrong disulfide bonds presumably occurs according to reactions 7 and 8, but the addition of low molecular weight thiols to the reactivation medium opens a new pathway, *via* reaction 9, for correction processes. However, it does not follow that in such systems, reactivation is speeded only by more rapid shuffling of disulfide bonds. Reexamination of eq 2–6 inclusive will show the complex involvement of RSH in the overall regeneration kinetics.

We have already shown that in the optimum range of thiol and disulfide concentrations, thiol–disulfide interchange reactions appear to be too fast to limit the regeneration of lysozyme. This not only rules out rate limitation by oxidation of protein SH groups, but also rules out rate limitation by

Scheme II



shuffling of disulfides according to reactions 7, 8, and 9. If any of these reactions were rate limiting under rapid regeneration conditions, the regeneration rate would depend on thiolate concentration. But the regeneration rate is independent of thiolate concentration. We have also presented evidence that the initial major chain-folding processes are completed so rapidly as to make no contribution to rate limitation of lysozyme regeneration. The question remains: What is the rate limiting step in lysozyme reactivation? We believe that it is the polypeptide backbone and side-chain folding that accompanies disulfide interchange reactions. For reactions 7, 8, and 9, it is reasonable to implicate chain folding as a component of the reaction in addition to the covalent chemistry explicitly appearing in these equations. Indeed it is difficult to imagine how these reactions could occur without concomitant chain folding. We may think of this as the second phase of chain folding, comprising minor but critical structural readjustments required for disulfide shuffling. It is also possible, although not as clearly necessary, that chain folding may accompany reactions 4, 5, and 6. This appears to be the simplest assignment of rate limitation that fits both the experimental facts and the conceptual framework.

Our optimal regeneration systems have a severalfold excess of thiol over the corresponding disulfide compound. This may at first appear surprising, that complete reoxidation of a disulfide-reduced protein should occur in a substantially reducing environment. There is no thermodynamic paradox here, because protein disulfides are generally much more stable to reduction than are low molecular weight, noncyclic disulfides (Bewley and Li, 1969). This greater stability of protein disulfides is linked, of course, to the stability of the whole protein molecule, or to large domains thereof.

It is relevant that protein biosynthesis occurs under substantially reducing conditions. Analysis of several mammalian

tissues for glutathione (Long *et al.*, 1961; Tietze, 1969) show total glutathione concentrations in the millimolar range, with GSSG:GSH ratios ranging from $^1/_{20}$ to $^1/_{100}$. Furthermore, the *in vitro* cell-free protein biosynthesizing systems appear to function optimally with either 10^{-2} M mercaptoethanol or 10^{-3} M dithiothreitol (Matthei and Nirenberg, 1961). If these are generally good indicators of the RSSR:RSH ratios at the sites of protein biosynthesis, then cysteinyl residues as they are incorporated into the growing polypeptide chain will be in the reduced form, until the chain grows to an extent that, by folding, it provides the additional free energy required for the stabilization of protein disulfide cross-links. This model clearly differs from that of Bradshaw *et al.* (1967), who have suggested that cysteine in the growing polypeptide chain may have its sulfur in disulfide linkage with low molecular weight thiol. If the biosynthetic milieu is as strongly reducing as present evidence indicates, very little of this kind of disulfide formation would occur. Our model is of course entirely different from the enzymic disulfide shuffling model of Anfinsen's group (Givol *et al.*, 1964).[3]

Considering that multiple reactions are involved in the regeneration process, the observed temperature effects are not presently understandable. It may be that in addition to the various chemical reactions outlined previously, formation and persistence of rate-limiting intermediates occurs optimally at 37°, but at present this is only a speculation.

Quite possibly biosynthetic chain folding is more rapid than our optimum *in vitro* rates of reactivation and may be as rapid as the rate of amino acid incorporation. It is also possible that chain folding can occur before completion of amino acid incorporation. There may well be differences in the rate and mode of folding for different proteins. For example, cross-linked proteins may have a different pattern of folding from those which are not cross-linked. Further, we recognize the possibility that biosynthetic folding may, at least in some cases, follow quite different kinetic pathways from those thus far obtained *in vitro*. We will attempt to answer some of these questions in further investigations.

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[3] We here quote the comments of one of the referees: "With regard to the authors' comments about the relationship of their model to that advanced by the group of Anfinsen, I think that the authors should consider the observations that protein synthesis often occurs in a compartmentalized situation. For those proteins being made within the cisternae of the endoplasmic reticulum, the presence of the disulfide interchange enzyme in the microsomes would probably be of considerably more importance than that of glutathione in the cell sap. Conversely, proteins being made on polysomes which are not membrane bound might be more susceptible to a system such as the authors discuss. In both situations, however, it should be stressed that *in vitro* studies are only a model for what is going on *in vivo*, and there may be many other variables affecting the rate of folding *in vivo* which cannot be taken into account in the model situation." The authors regard the foregoing comments about possible compartmentallization of the intracellular oxidoreduction systems as plausible but unproven.

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Extracellular 2-Acetamido-2-deoxy-D-galacto-D-galactan from *Aspergillus nidulans**

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ABSTRACT: An extracellular polysaccharide from *Aspergillus nidulans* is a linear molecule containing 4-O-substituted α-D-galactopyranosyl and 4-O-substituted 2′-acetamido-2′-deoxy-α-D-galactopyranosyl units in an approximately 1.8:1 ratio. A Smith degradation yielded mainly 2-*O*-(2′-acetamido-2′-deoxy-α-D-galactopyranosyl)-D-threitol, two hydroxyethylidene acetals, D-threitol, and a trace of unidentified material. The different sugar units are distributed evenly throughout the chain, consecutive *N*-acetyl-D-galactosamino units not being detected in appreciable amounts. 3,4,6-Tri-*O*-methyl-D-galactose was prepared for use of its methyl glycoside as a gas–liquid chromatographic standard.

Initial tests on an extracellular polysaccharide produced by *Aspergillus nidulans* indicated it to be a comparatively rare fungal polymer of a galactosamine and a galactose; a sugar composition which has only once been found in a fungal polysaccharide (Trotter and Whisler, 1965). There are important relationships between cell wall (especially polysaccharide structure) and both fungal taxonomy and morphogenesis (recently reviewed by Bartnicki-Garcia (1968)) and thus a more detailed examination of the *Aspergillus* heteropolymer was carried out.

Experimental Procedure

Production of Polysaccharide. Aspergillus nidulans (Eidam) Wint. strain b 1 was supplied by M. Reever, Department of Biology, University of Saskatchewan, *via* G. Pontecorvo, Glasgow. The organism was routinely grown on Vogel's medium N (Vogel, 1964) with 1% D-glucose as a carbon source. Alternatively a medium (M. J. Johnson, personal communication) was used consisting of D-glucose, 10 g/l.; KH_2PO_4, 5.5 g/l.; $MgSO_4 \cdot 7H_2O$, 0.2 g/l.; $CaCl_2$, 0.015 g/l.; $(NH_4)_2HPO_4$, 2.0 g/l.; $(NH_4)H_2PO_4$, 1.5 g/l.; Na_2HPO_4, 10.0 g/l.; $Fe_2(SO_4)_3$, 0.6 mg/l.; $ZnSO_4 \cdot 7H_2O$, 0.2 mg/l.; $CuSO_4 \cdot 5H_2O$, 0.2 mg/l.; $MnSO_4 \cdot H_2O$, 0.2 mg/l.; biotin, 10 μg/l. Cultures were grown at 30° in erlenmeyer flasks (*e.g.*, 900 ml in each 2-l. flask) on a rotary shaker. The mycelium was filtered off using cheese cloth and the extracellular polysaccharide precipitated from the culture fluid by the addition of 1 volume of ethanol. Further soluble material could be obtained by washing the mycelium with water. A polysaccharide of a different type was precipitated on standing overnight at 5°.

General Procedures. Descending paper chromatography was carried out on Whatman No. 1 filter paper using the following solvent systems (in volume ratios): (A) butyl alcohol–ethanol–water (40:11:19), (B) 2-propanol–water (4:1), (C) ethyl acetate–acetic acid–water (9:2:2), (D) ethyl acetate–pyridine–water (10:4:3), (E) isoamyl alcohol–pyridine–water (5:5:4) (Powning and Irzykiewicz, 1965). Gas chromatography of methyl glycosides was carried out on a 12 ft × 0.25 in., outside diameter, 3% neopentylglycol succinate on Chromosorb W column maintained at 170° using helium (40 psi) as carrier gas (Lee and Ballou, 1965), while amino sugars were also characterized on a Spinco amino acid analyzer, on paper chromatograms, and by gas–liquid chromatography of their trimethylsilyl derivatives (P. A. J. Gorin, data to be published). Threitol was characterized by gas–liquid chromatography of its tetraacetate using a column containing a nitrile silicone XE-60 packing (Gorin and Spencer, 1966).

Sugars were detected on paper chromatograms with *p*-anisidine hydrochloride (Hough *et al.*, 1950), ammoniacal silver nitrate (Partridge, 1946), ninhydrin, and Elson–Morgan dimethylaminobenzaldehyde (Partridge, 1948) spray reagents.

Acid hydrolyses of the polysaccharide were carried out in 3 M HCl at 100° for 2 hr and the solution evaporated, or in 1 M H_2SO_4 at 100° for 18 hr and the solution was neutralized ($BaCO_3$), filtered, and evaporated. Partial acid hydrolysis was performed on the dispersed polysaccharide on 0.15 M HCl, at 100° for 30 min. The mobile solution was deionized with mixed Amberlite 1R120 (H^+ form) and Dowex 1-X8 (bicarbonate form) and the polysaccharide precipitated with excess ethanol.

Methylation was carried out by the successive procedures of Haworth (1915) and Kuhn *et al.* (1955). Proton magnetic resonance spectra were taken using a Varian HA-100 high-resolution nuclear magnetic resonance spectrometer. Measurements were made in D_2O at 70° with tetramethylsilane in a coaxial capillary as external standard (τ 10).

Preparation of 3,4,6-Tri-O-methyl-D-galactose. 1,2,3,4,6-Penta-*O*-acetyl-β-D-galactose (5 g) was converted into

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