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Pathways of Folding of Reduced Bovine Pancreatic Ribonuclease†

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ABSTRACT: The reoxidation of reduced ribonuclease under anaerobic conditions was found to be accelerated by a mixture of reduced and oxidized glutathione. At pH 7.65 and 23–24°, optimal rates of activity regain occurred with equimolar concentrations of reduced and oxidized glutathione of 5×10^{-3} M. The rate of reoxidation was studied by following the accompanying changes in enzymatic activity, absorbance, fluorescence, and sulfhydryl group titer. The time dependence of the activity regain could be described by a single first-order rate constant of 7×10^{-3} min^{-1}. However, the time dependence of the changes in absorbance and fluorescence accompanying reoxidation obeyed a rate equation with two first-order exponential terms. The larger rate contant had a value of approximately 0.1 min^{-1}, while the smaller rate constant was similar to the one characterizing the regain of enzymatic activity. An initial very rapid change in fluorescence, too rapid for kinetic studies, also was observed. Disulfide bond formation was found to take place in two steps: a fast step in which the number of free sulfhydryl groups decreased from 8 to about 1, followed by a much slower decrease until essentially no free sulfhydryl groups existed. Measurement of the formation of mixed disulfide bonds between reduced ribonuclease and glutathione indicated that almost all of the disulfide bonds that were formed *after* the initial rapid change in sulfhydryl group titer were within the ribonuclease molecule. The time course of the reoxidation was also monitored by peptide mapping: the results obtained indicate the initial formation of a large number of incorrect, *i.e.*, nonnative pairings of cysteine residues, followed by a slower rearrangement to the covalent structure of the native enzyme. The rates of renaturation of ribonuclease denatured with guanidine hydrochloride were measured to ascertain the refolding rate with the disulfide bonds intact. A mechanism for reoxidation is proposed involving formation of mixed disulfides between glutathione and reduced enzyme followed by rapid formation of a large number of different inactive species with incorrectly paired cysteine residues. These incorrectly paired species then undergo a conformational change and glutathione-catalyzed disulfide exchange reactions which ultimately lead to the native conformation. A A small amount of the enzyme (about 10%) was not renatured and apparently remains in a nonnative conformation.

The mechanism for folding newly synthesized proteins into biologically active molecules is an important biological problem not yet understood in molecular detail. Its elucidation is the subject of much current experimental and theoretical work. One experimental approach is to perturb the native structure of a protein with some denaturing reagent, either with or without the disruption of disulfide bonds, and to study the *in vitro* unfolding or the refolding to the original structure.

Ribonuclease is a small protein, whose known three-dimensional structure (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967) is stabilized by four intrachain disulfide bonds. These can be reduced in 8 M urea by an excess of 2-mercaptoethanol, and the protein then assumes a random coil conformation (White, 1961). At pH 8 in the presence of oxygen, the cysteine residues of the reduced protein can be oxidized to disulfide bonds, and the protein returns to its original three-dimensional structure (Anfinsen *et al.*, 1961). Air oxidation is a rather inefficient process, and the rate of reoxidation apparently depends on trace amounts of divalent metal ions (unpublished results). Recently, the reoxidation of reduced lysozyme has been found to be accelerated by a mixture of reduced and oxidized glutathione, presumably by acceleration of disulfide exchange (Saxena and Wetlaufer, 1970).

In the work presented here, the reoxidation of reduced ribonuclease has been studied under anaerobic conditions in the presence of reduced and oxidized glutathione, in order to elucidate the mechanism of the renaturation process and the role of disulfide bond formation in the refolding. The time course of the reoxidation of reduced ribonuclease was followed by measuring the changes in enzymatic activity, absorbance, fluorescence, and sulfhydryl group titer which accompany renaturation. The extent of formation of mixed disulfide bonds with glutathione was also measured. To aid in the correlation of spectral, chemical, and structural properties, peptide maps which indicate the distribution of disulfide bonds during reoxidation were also obtained.

The results of these experiments indicate that the reoxida-

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tion proceeds by rapid formation of a large number of different structures with incorrectly paired cysteine residues, followed by a slower rearrangement of the disulfide bonds to produce an active enzyme. A mechanism is proposed which is consistent with the measured rates of renaturation of native ribonuclease, denatured with guanidine hydrochloride, and the known rates of disulfide exchange reactions in model systems. The native structure apparently can be reached from many different structures with incorrectly paired cysteine residues; this indicates that the intervening conformational space provides many accessible pathways to the native conformation.

Experimental Section

Materials. Glutathione (reduced and oxidized), *N*-ethylmaleimide, ninhydrin, ε-Dnp-lysine, and *N*-Dnp-glutamic acid purchased from Sigma, 2,4-dinitrophenol purchased from Eastman, and ultrapure guanidine hydrochloride purchased from Schwarz Mann were used without further purification. *p*-Hydroxymercuribenzoate was purchased from Sigma and was purified by acid precipitation from sodium hydroxide solutions (Boyer, 1954). Urea and sodium dodecyl sulfate were obtained from Fisher and were rerecrystallized from ethanol. Trichloroacetic acid, from Mallinckrodt, was recrystallized twice from benzene and three times from chloroform. Fisher reagent grade pyridine was distilled from ninhydrin (Benson *et al.*, 1966). Carbon-14 labeled *N*-ethylmaleimide (10 Ci/mol) was purchased from SchwarzMann. Tritium labeled reduced glutathione (240 Ci/mol) was purchased from New England Nuclear. The ribonuclease (Code RAF) and the pepsin (Code PM) were purchased from Worthington and were used without further purification. Cytidine 2′,3′-cyclic phosphate was synthesized as previously described (Crook *et al.*, 1960a). Whatman 3MM chromatography paper was purchased from Fisher.

Fully reduced ribonuclease was prepared according to the method of White (1967) with the following modifications: Tris-chloride buffer, 0.1 M (pH 9), was used in place of trimethylamine, the G-25 Sephadex separation was performed at 4°, and the product was lyophilized and stored *in vacuo* or under nitrogen at −10°. This preparation was stable for several weeks without significant change in the number of sulfhydryl groups per molecule and was found to have no enzymatic activity with cytidine 2′,3′-cyclic phosphate as substrate (*cf.* Crook *et al.*, 1960b).

The number of sulfhydryl groups per enzyme molecule was determined by spectrophotometric titration with *p*-hydroxymercuribenzoate at pH 7 (Boyer, 1954). The difference extinction coefficient at 250 nm between reduced ribonuclease and the product of the reaction with *p*-hydroxymercuribenzoate and reduced ribonuclease was determined to be $8.88 \pm 0.11 \times 10^3$ cm^{-1} M^{-1}. Spectrophotometric titrations were also performed with *N*-ethylmaleimide at pH 6 (Alexander, 1958). The number of sulfhydryl groups per molecule, measured with both methods, was in the range of 7.5–8.0 indicating essentially complete reduction of ribonuclease.

Protein Concentration. The concentration of native and reduced ribonuclease solutions was determined specrophotometrically with a Zeiss Model PMQ II spectrophotometer using the following molar extinction coefficients: 9800 M^{-1} cm^{-1} for native ribonuclease at 277.5 nm (Harrington and Schellman, 1956), 9390 M^{-1} cm^{-1} for fully reduced ribonuclease at 276 nm (White, 1961), and 9330 M^{-1} at 275 nm, the isosbestic wavelength for native and fully reduced enzyme (measured; literature value 9200 M^{-1} cm^{-1} (Anfinsen *et al.* 1961)).

The concentration of fully reduced ribonuclease, reacted with *N*-ethylmaleimide to eliminate interference by thiols, was determined by the method of Lowry *et al.* (1951), using native ribonuclease as a standard. The concentration of the enzyme–*p*-hydroxymercuribenzoate adduct was determined by the micro-Kjehldahl method (Ballantine, 1957) using native enzyme as a standard.

Spectrophotometric and pH Measurements. The pH measurements were made on a Radiometer Model 26 pH meter with a combination glass–calomel electrode. Since thiols interfere with pH measurements (Hill and Spivey, 1974), narrow range pH paper was used as a check when the solutions contained glutathione.

A Zeiss Model PMQ II spectrophotometer was used for determinations of difference spectra.

Fluorescence was measured using a Hitachi-Perkin-Elmer spectrofluorimeter, Model MPF-3, with an excitation wavelength of 275 nm and an emission wavelength of 303 nm. The fluorescence emission was linear with protein concentration up to 2×10^{-5} M ribonuclease in square cross-section cells (1 cm × 1 cm) and up to 5×10^{-5} M in triangular cells. Fluorescence measurements were made at 25.0°.

The optical rotary dispersion of ribonuclease was measured at 25.0° using a Cary Model 60 spectropolarimeter over the wavelength range 300–600 nm.

Kinetic Methods. Solutions of native ribonuclease (1.8×10^{-5}–1.3×10^{-4} M) in 4.0 M guanidine hydrochloride–0.1 M imidazole chloride (pH 7.0) were diluted with buffer to a final guanidine hydrochloride concentration of 2.0 or 1.3 M. The renaturation was followed by measuring the change in absorbance at 287 nm in a Cary Model 14 spectrophotometer thermostated at 25.0°. Similar renaturation experiments were carried out by measuring the change in optical rotation of ribonuclease solutions (4.7×10^{-4} M) at 360 nm.

The reoxidation of reduced enzyme was carried out in the presence of reduced and oxidized glutathione; this system is similar to that used in a study of the reoxidation of reduced lysozyme (Saxena and Wetlaufer, 1970). To exclude air oxidation, the reactions were carried out in deaerated, nitrogen saturated solutions. For the initial measurements of activity, a solution of reduced ribonuclease and reduced and oxidized glutathione (pH 3) was prepared and degassed by bubbling nitrogen through the solution for 15 min. A separate solution of concentrated Tris-chlride buffer (pH 8.0) was similarly degassed. To start the reaction, the two solutions were mixed to raise the pH to 7.6–7.9, nitrogen was blown into the test tube, and a stopper was inserted. The reaction was carried out at 23–24° for 60 min, after which time an aliquot was removed and its activity (relative to a ribonuclease standard) determined.

The initial measurements of activity (after 60 min of reoxidation) were used to determine the optimum conditions for reoxidation of reduced ribonuclease. These were followed by measurements over the complete time course of reoxidation; these latter experiments allowed the determination of rate constants for the reoxidation. For this purpose, a special two-compartment reaction cell consisting of two 50-ml round-bottom flasks, joined by a glass tube, was used. The solution of reduced enzyme and glutathione (reduced and oxidized forms), pH 3, was placed in one compartment and 1.0 M Tris-chloride (pH 8.0) in the other. The cell contents were deaerated by freezing and thawing under vacuum (Blackburn, 1968), using sonication to aid deaeration during the thawing step, and the solutions were equilibrated with nitrogen in a final step. The cell was tilted to mix the components without exposure to the air; this raised the pH to 7.6–7.9 and started the reaction in less than 15 sec. Aliquots of the reaction mixture were removed through a serum cap with a Hamilton gas tight syringe. In

order to determine the efficiency of this deaeration procedure, control measurements of the oxidation of reduced glutathione and the reoxidation of reduced ribonuclease in the absence of glutathione at pH 8 were made. The results indicated that a negligible amount of air oxidation of reduced ribonuclease and glutathione had occurred in 200 min.

To determine the enzymatic activity during reoxidation, an aliquot of the reoxidation reaction mixture was removed from the cell and diluted into a cuvet containing cytidine 2',3'-cyclic phosphate at 0.1 mg/ml in 0.1 M Tris-chloride (pH 7.0) at 25.0°. The change in absorbance at 288 nm was monitored on a Cary Model 14 spectrophotometer. The activity, expressed as the change in absorbance per unit time, was found to be linear in ribonuclease concentration from 2.0×10^{-8} to 2.0×10^{-6} M. For the initial determinations of the activity regain, the concentrations of reduced and oxidized glutathione were varied, and the amount of activity regained at 60 min was determined. For the more detailed kinetic measurements, samples were withdrawn from the reaction mixture at various times during the course of the reaction and their activity relative to a ribonuclease standard was determined. In these measurements, the concentration of enzyme was $6.1\text{-}7.0 \times 10^{-5}$ M, and glutathione (reduced and oxidized) was present in equimolar concentrations of 5×10^{-4} M at pH 7.92 and 5×10^{-3} M at pH 7.65. The lower glutathione concentrations were used in some experimnts in order to make a direct comparison between the activity measurements and the fluorescence measurements, which are done at the lower glutathione concentrations to reduce quenching.

To follow the changes in absorbance during reoxidation, the reaction mixture was prepared as described previously, and an aliquot of the solution was transferred to a quartz cell fitted with a gas-tight connection to a nitrogen tank. A Zeiss Model PMQ II spectrophotometer thermostated at 25.0° was used. The absorbance at 275 nm, an isosbestic point for native and fully reduced ribonuclease, and at 287 nm was measured in order to separate the spectral changes due to glutathione oxidation from those due to ribonuclease reoxidation. The measurements were made in 0.1 M Tris-chloride buffer (pH 7.65) with equimolar concentrations of reduced and oxidized glutathione of 4.5×10^{-3} M and an enzyme concentration of 1.10×10^{-4} M.

To determine the changes in fluorescence during reoxidation, the reaction mixture was prepared as usual and transferred to a cell fitted to a nitrogen tank. An excitation wavelength of 275 nm (slit width 2 nm) and an emission wavelength of 303 nm (slit width 10 nm) were used with a Perkin-Elmer-Hitachi spectrofluorimeter thermostated at 25.0°. The reaction mixture consisted of 0.1 M Tris-chloride buffer (pH 7.92) with equimolar concentrations of reduced and oxidized glutathione of 5×10^{-4} M. (The lower glutathione concentrations were necessary to reduce fluorescence quenching.) The ribonuclease concentration was varied from 5.4×10^{-6} to 5.8×10^{-5} M.

Two procedures were used to determine the sulfhydryl group titer during reoxidation. The problem is complicated by the reduced glutathione present in the reaction mixture. In the first procedure, a sample of the reoxidation mixture was withdrawn and mixed with an equal volume of 40% trichloroacetic acid which quenches the oxidation process. This precipitates the ribonuclease, which is centrifuged, washed twice with 20% trichloroacetic acid, and redissolved in 1% sodium dodecyl sulfate (pH 4). Portions of this solution were used for sulfhydryl group analysis with *p*-hydroxymercuribenzoate (Boyer, 1954) at pH 7. The protein concentration was determined by a micro-Kjehldahl analysis (Ballantine, 1957). In the second procedure, a sample of the reaction mixture was removed, its pH was adjusted to 6 with imidazole-chloride buffer, and sufficient ^{14}C-labeled *N*-ethylmaleimide (two- to threefold excess) was added to react with all protein and glutathione thiols. The resultant enzyme-ethylmaleimide adduct was separated from glutathione on a G-25 Sephadex column. The radioactivity was determined by addition of the adduct to Bray's scintillation fluid (Bray, 1960) and measuring the radioactivity on a Beckman Model LS-255 scintillation counter. The protein concentration was measured by the method of Lowry *et al.* (1951). The specific activity of the *N*-ethylmaleimide solution was determined by reaction with fully reduced ribonuclease with no glutathione present, followed by chromatographic separation of the protein and radioactive counting; the specific activity of the protein sulfhydryl-ethylmaleimide adduct was 5.6 mCi/mol. The reaction mixture consisted of equimolar concentrations of reduced and oxidized glutathione of 5×10^{-3} M in 0.1 M Tris-chloride buffer (pH 7.65) with protein concentrations in the range $5\text{-}7 \times 10^{-5}$ M. The reaction was carried out at room temperature, 23-24°.

As the possibility exists that some of the free sulfhydryl groups of reduced ribonuclease form mixed disulfide compounds with the oxidized glutathione present in the reoxidation mixture, experiments to ascertain the extent of this protein-glutathione interaction were carried out with radioactively labeled glutathione. The purity of the radioactive glutathione was determined by reacting the glutathione with *N*-ethylmaleimide, followed by paper chromatography of the adduct formed using the solvent system 1-butanol (70%)-formic acid (20%)-water (10%) (Gutcho and Laufer, 1954). The chromatogram was cut into strips which were put into scintillation fluid (Bray, 1960) and their radioactivity was determined. Greater than 65% of the radioactivity was found at the R_F of the glutathione-*N*-ethylmaleimide adduct (Gutcho and Laufer, 1954). Tritium labeled reduced glutathione (specific activity 2.9×10^5 mCi/mol) was converted to the corresponding disulfide (oxidized glutathione) by air oxidation at pH 8. The reduced and oxidized compounds were used to prepare a reoxidation mixture containing equimolar reduced and oxidized glutathione concentrations of 5×10^{-3} M (specific activity 72 mCi/mol) in 0.1 M Tris-chloride (pH 7.65). The reduced ribonuclease concentration was 5×10^{-5} M and the reoxidation reaction was carried out at room temperature, 23-24°. At 0, 1, and 5 min of reoxidation, aliquots of the reaction mixture were removed and precipitated with trichloroacetic acid, centrifuged, washed, and dissolved in sodium dodecyl sulfate. The extent of glutathione attachment to native and fully reduced enzyme, as well as to enzyme during reoxidation, was measured by determination of the radioactivity of the protein in Bray's scintillation fluid (Bray, 1960). The protein concentrations were determined by micro-Kjehldahl analysis. These experiments indicate the extent of glutathione attachment, but not the mode of attachment of glutathione to the enzyme (*i.e.*, covalent or noncovalent).

Peptide Mapping. The diagonal electrophoresis technique (Brown and Hartley, 1966) was used to identify the disulfide bonds of ribonuclease formed during reoxidation. The procedure used involved digestion of enzyme at 5 mg/ml with pepsin at 0.03 mg/ml in 5% formic acid at 37° for 20 hr. After lyophilization, 1.5-4.5-mg samples were dissolved in 10-20 μl of pyridine-acetic acid-water buffer and applied to a 17 in. × 17 in. Whatman 3MM paper. Electrophoresis was carried out with pyridine-acetic acid-water (25:1:225) buffer (pH 6.5) at 58 V/cm for 3 hr at 12° on a modified Savant Instrument Co. flat plate apparatus. The modifications, which were designed to en-

sure uniform wetness and cooling of the paper, included cellophane barriers for wicks, a Lucite cover with machined grooves to accommodate the extra thickness of wicks and stitches, and a pneumatic device to keep a constant pressure of 5 psi on the paper during electrophoresis.

After the separation in the first dimension, performic acid oxidation was carried out for 2 hr (Brown and Hartley, 1966). The strip was then sewn to a clean sheet of paper for separation in the second dimension at 35 V/cm for 2.5 hr in the same buffer (cooling is less efficient at the sewn strip necessitating lower power).

The spots were developed with ninhydrin (Easley, 1965), and the mobilities were measured relative to 2,4-dinitrophenol, which has a mobility of 0.65 compared to aspartic acid. Correction for endoosmosis was made by using the position of the neutral spot, ϵ-Dnp-lysine, as the origin (Stevenson, 1971). To identify the individual off diagonal spots, duplicate electrophoretograms were prepared, and one was developed with ninhydrin. This served as a template to locate the undeveloped spots in the second chromatogram. The undeveloped spots were cut out and eluted with 50% pyridine, water, and 1 M acetic acid (Gall and Edelman, 1970). The peptides were hydrolyzed in sealed tubes under reduced pressure in constant-boiling HCl for 20 hr at 105°, and the hydrolysate was dried by rotary evaporation.

Amino acid analyses were carried out on a Beckman Model 116 autoanalyzer, with individual samples of about 0.01 μmol. Since this is near the limit of detection, the color constants for each amino acid were determined on a 0.01-μmol standard. The values obtained were within the standard deviation of the larger (0.1 μmol) standards.

To follow the time course of reoxidation, a reaction mixture of 5×10^{-3} M reduced and oxidized glutathione and fully reduced enzyme at 1 mg/ml in 0.1 M Tris-chloride buffer (pH 7.65) was prepared. The reaction was carried out in nitrogen-saturated solutions at room temperature, 23–24°. Samples of 5 ml were removed at various times, the pH was quickly adjusted to 6 with imidazole-chloride buffer, and an excess of *N*-ethylmaleimide was added to block all free sulfhydryl groups and prevent disulfide interchange. The protein was separated from glutathione on a G-25 Sephadex column, lyophilized, and digested with pepsin, and the peptide map was obtained and compared to that of native enzyme.

Results

Renaturation from Guanidine Hydrochloride. In order to obtain a measure of the rate of refolding of the denatured enzyme not accompanied by disulfide bond formation, the rate of renaturation from guanidine hydrochloride was determined. With the native enzyme in 0.1 M imidazole chloride (pH 7.0), a plot of the difference extinction coefficient at 287 nm, $\Delta\epsilon_{287}$, shows a sharp reversible transition in the 2–4 M guanidine hydrochloride range with a midpoint at 3.0 M guanidine hydrochloride. (This is similar to the results of Sela and Anfinsen (1957).) The maximum value of $\Delta\epsilon_{287}$ is -2450 ± 90 M^{-1} cm^{-1} at room temperature, 23–24°. (This difference coefficient has not been corrected for solvent effects on the tyrosine absorption spectrum.)

The rate of renaturation (with disulfide bonds intact) in going from 4.0 to 2.0 M guanidine hydrochloride over a range of ribonuclease concentrations (2.8×10^{-5}–1.3×10^{-4} M) in 0.1 M imidazole chloride (pH 7.0) can be characterized by a first-order rate constant of 0.418 ± 0.023 min^{-1}, as measured by ultraviolet absorbance changes at 287 nm. At 4.7×10^{-4} M enzyme, the rate constant measured by following changes in optical rotation at 360 nm is 0.411 ± 0.031 min^{-1}. The absorbance changes at 287 nm accompanying the transition from 4.0 to 1.3 M guanidine hydrochloride are characterized by a rate constant of 0.772 ± 0.010 min^{-1} at protein concentrations of 1.85–4.0×10^{-5} M. Although a complete study was not carried out, these results indicate that the rate constants depend on the final concentration of guanidine hydrochloride.

TABLE I: Dependence of Reduced Ribonuclease Reoxidation on Glutathione Concentrations.[a]

Ribonuclease (M)	Glutathione (M) (reduced)	Glutathione (M) (oxidized)	% Activity Regain at 60 min
1.3×10^{-4}	5.0×10^{-3}	5.0×10^{-4}	13.2
1.6×10^{-5}	5.0×10^{-3}	5.0×10^{-4}	12.6
1.3×10^{-4}	5.0×10^{-3}	5.0×10^{-3}	15.9
1.6×10^{-5}	5.0×10^{-3}	5.0×10^{-3}	13.1
1.3×10^{-4}	2.5×10^{-2}	5.0×10^{-3}	0.0
1.6×10^{-5}	2.5×10^{-2}	5.0×10^{-3}	1.8
8.3×10^{-5}	0.0	4.5×10^{-3}	8.2
8.3×10^{-5}	9.8×10^{-5}	4.5×10^{-3}	8.0
7.4×10^{-5}	5.0×10^{-4}	4.0×10^{-3}	11.6

[a] 0.1 M Tris-chloride (pH 7.6–7.9).

The total changes in absorbance observed in the kinetic measurements were comparable to those measured by static methods, indicating that no reactions faster than those associated with the measured rate constants are occurring in the refolding process. Stopped flow experiments designed to measure rapid transients failed because of spurious effects from the mixing of viscous solutions. Although the absorbance of ribonuclease solutions in 0.1 M imidazole chloride buffer (pH 6) and 2.8 M guanidine hydrochloride was temperature dependent, temperature jump experiments in the range 20–30° did not reveal any fast rate processes. Therefore, a single first-order process adequately describes all of the renaturation data.

Reoxidation of Reduced Ribonuclease. The influence of the concentrations of reduced and oxidized glutathione on the regain of enzyme activity during the oxidation of reduced ribonuclease was studied, and the results obtained are summarized in Table I. The optimal concentration of reduced and oxidized glutathione in 0.1 M Tris-chloride buffer (pH 7.6–7.9) is an equimolar mixture of about 5×10^{-3} M. The rate of regain of activity is not dependent on the protein concentration over a range of ribonuclease concentration from 1.6×10^{-5} to 1.3×10^{-4} M. The regain of activity of enzyme during 2 half-lives of oxidation was measured, and a typical set of data i shown in Figure 1, where the fraction of the maximum observed activity is plotted *vs.* time. The data were fit to an equation of the form

$$y(t) = y(\infty)(1 - e^{-k_2 t}) \quad (1)$$

by a nonlinear least-squares analysis. A short induction period may occur before the activity begins to increase, but the experimental precision is not sufficient to establish this with certainty. The value of $y(\infty)$ was determined from the data and is equal to the activity which would be obtained at infinite time. The values of $y(\infty)$ and k_2 did not depend significantly on the glutathione concentration over a range 5×10^{-4}–5×10^{-3} M glutathione (equimolar amounts of reduced and oxidized forms). The average rate constant, determined from three experiments, is cited in Table II. The value of $y(\infty)$, relative to an identical concentration of native ribonuclease, is 87 ± 8%. These reoxidation experiments were carried out in 0.1 M Tris-

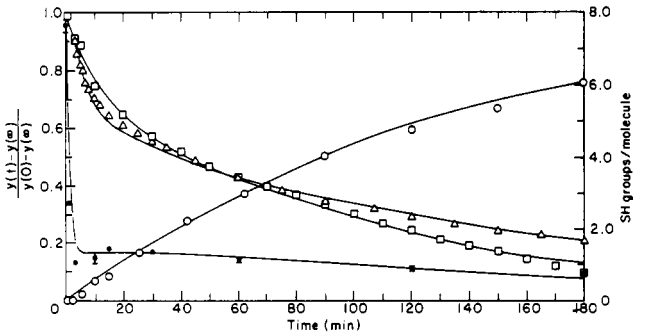


FIGURE 1: Time dependence of the reoxidation of reduced ribonuclease. The left-hand ordinate is $[y(t) - y(\infty)]/[y(0) - y(\infty)]$ where y is the activity (○), absorbance (□), or fluorescence (△). The experimental conditions are as follows: activity, 0.1 M Tris-chloride (pH 7.92), 5×10^{-4} M reduced and oxidized glutathione, 6.1×10^{-5} M ribonuclease, 23–24°; absorbance, 0.1 M Tris-chloride (pH 7.65), 5×10^{-3} M reduced and oxidized glutathione, 1.1×10^{-4} M ribonuclease, 25.0°; and fluorescence, 0.1 M Tris-chloride (pH 7.92), 5×10^{-4} M reduced and oxidized glutathione, 5.8×10^{-5} M ribonuclease, 25.0°. The solid lines have been calculated with eq 1 and 6 using the parameters in Table II. The right ordinate is the number of sulfhydryl groups per protein molecule (●). The bars indicate the average of the results from the *p*-hydroxymercuribenzoate and *N*-ethylmaleimide methods of determining the sulfhydryl titer. The experiments were carried out in 0.1 M Tris-chloride (pH 7.65), 5×10^{-3} M reduced and oxidized glutathione, $5\text{–}7 \times 10^{-5}$ M ribonuclease at 23–24°. The abscissa in all cases is the time in minutes.

chloride buffer (pH 7.65–7.92) at room temperature, 23–24°. The protein concentration was $6.0\text{–}7.1 \times 10^{-5}$ M.

The difference extinction coefficient, $\Delta\epsilon_\lambda$, between native enzyme in water and reduced enzyme in 0.001 M HCl exhibits features characteristic of the unfolding of the protein with the disulfide bonds intact: $\Delta\epsilon_{287} = -2650$, $\Delta\epsilon_{279} = -1090$, and $\Delta\epsilon_{275} \simeq 0$ M^{-1} cm^{-1}. The time dependence of the absorbance during reoxidation is complicated by the fact that the oxidation of reduced glutathione is also accompanied by absorbance changes. However, 275 nm is an isosbestic point for reduced and native enzyme, so that changes in absorbance at this wavelength arise only from oxidation of glutathione. On the other hand, changes in absorbance at 287 nm arise from oxidation of both protein and glutathione. The absorbance of reduced glutathione is negligible at both wavelengths, so that the absorbance, A, is given by

$$A_{287} = \epsilon_G[G] + \epsilon_R[R] + \epsilon_N[N] \quad (2)$$

$$A_{275} = \epsilon_G'[G] + \epsilon_R'[R] + \epsilon_N'[N] \quad (3)$$

where G is oxidized glutathione, R is reduced enzyme, N is native enzyme, ϵ and ϵ' are the corresponding extinction coefficients at 287 and 275 nm, respectively, and $\epsilon_R' = \epsilon_N'$. The concentration of oxidized glutathione can be eliminated from eq 3 with eq 2 to give eq 4, in which $[R_0]$ is the initial concentration

$$A_{287} = (\epsilon_G/\epsilon_G')(A_{275} - \epsilon_{R,N}'[R_0]) + \epsilon_R[R_0] + \Delta A \quad (4)$$

of reduced ribonuclease and $\Delta A = (\epsilon_N - \epsilon_R)[N]$. The measured values of the extinction coefficients are as follows: $\epsilon_G = 66$ M^{-1} cm^{-1}, $\epsilon_G' = 134$ M^{-1} cm^{-1}, $\epsilon_R' = \epsilon_N' = 9330$ M^{-1} cm^{-1}, $\epsilon_N = 6630$ M^{-1} cm^{-1}, and $\epsilon_R = 3980$ M^{-1} cm^{-1}. Thus eq 4 can be rewritten as

$$\Delta A = A_{287} - 0.493A_{275} + 0.067 \quad (5)$$

The values of the changes in absorbance at 275 and 287 nm during reoxidation were plotted against time, and the time dependence of ΔA was determined by use of eq 5. A typical set of data is shown in Figure 1. The time dependence of ΔA, which is proportional to the concentration of renatured enzyme, cannot be described by a single exponential function. The simplest equation which describes the data is

$$[y(t) - y(\infty)]/[y(0) - y(\infty)] = ae^{-k_1 t} + be^{-k_2 t} \quad (6)$$

in which y corresponds to ΔA, and a/b, k_1, and k_2 are parameters. The value of $\Delta A(\infty)$ was estimated to be −0.42 from the extrapolation of the experimental curve to long times. The value predicted from the estimated protein concentration is −0.3. This difference may be due to an increase in protein concentration during the deaeration step, or to cumulative errors involved in calculating ΔA from the small difference in two larger numbers. Equation 6 was fit to the data by a nonlinear least-squares analysis. The rate constants and amplitude parameters obtained are given in Table II. The values of these parameters depend on the choice of $\Delta A(\infty)$, so that the error limits obtained from the statistical analysis are undoubtedly an underestimate. The absorbance measurements were carried out in 0.1 M Tris-chloride (pH 7.65) at 25.0° with a protein concentration of 1.10×10^{-4} M and equimolar concentrations of reduced and oxidized glutathione of 4.5×10^{-3} M.

The fluorescence emission at 303 nm (excitation 275 nm) of native and denatured ribonuclease also differs. With entrance and exit slits at 10 nm, the fluorescent intensity of native enzyme in 0.1 M Tris-chloride (pH 7.0)–4 M guanidine hydrochloride is 2.3 times that of native enzyme in the absence of guanidine hydrochloride. The value for fully reduced enzyme in 0.1 M acetic acid is 3.3 times that of the native enzyme; the addition of 4 M guanidine hydrochloride increases the ratio to 3.5.

The time dependence of the fluorescence during reoxidation could not be fit to a single exponential, but eq 6 describes the data well, if y is expressed in arbitrary fluorescence units. A typical set of data is shown in Figure 1. The value of $y(\infty)$ is estimated from the fluorescent intensity of an equivalent concentration of native ribonuclease, and the three parameters of eq 6 were obtained by a nonlinear least-squares analysis. The rate constants and the ratio a/b did not depend on protein concen-

TABLE II: Rate Constants for Reoxidation of Reduced Ribonuclease in 0.1 M Tris-Chloride.

Method	k_1 (min^{-1})	k_2 (min^{-1})	a	b
Activity[a]		$6.6 \pm 1.7 \times 10^{-3}$		
Absorbance[b]	0.130 ± 0.029	$9.9 \pm 0.4 \times 10^{-3}$	0.22 ± 0.03	0.78 ± 0.02
Fluorescence[c]	0.145 ± 0.062	$5.6 \pm 1.2 \times 10^{-3}$	0.39 ± 0.05	0.59 ± 0.03
Sulfhydryl titer[d]	Half-time <1 min			

[a] pH 7.65 and pH 7.92, 23–24°. [b] pH 7.65, 25.0°. [c] pH 7.92, 25.0°; a very rapid process with a half-time of less than 30 sec is also observed. [d] pH 7.65, 23–24°.

TABLE III: Amino Acid Analyses of Spots on Peptide Map of Native Ribonuclease and Assignment to Specific Sequences.[a]

Amino acid sequence	Residues 110–120			Residues 56–76			Residues 78–96		
Half-cystines	VIII			III, IV, V			VI, VII		
μ(obsd)[b]	(0.18 ± 0.02, −0.46 ± 0.08)[b]			(0.18 ± 0.02, −0.13 ± 0.05)			(0.44 ± 0.02, −0.52 ± 0.02)		
μ(calcd)	(0.21, −0.54)[b]			(0.21, −0.25)			(0.56, −0.52)		
	Spot 1			Spot 2			Spot 3		
Amino Acid	μmol	Calcd stoic.	Assumed stoic.	μmol	Calcd stoic.	Assumed stoic.	μmol	Calcd stoic.	Assumed stoic.
Lysine				0.015	1.8	2	0.0078	1.0	1
Histidine	0.0057	1.2	1						
Arginine							0.0073	0.96	1
Cysteic acid	0.0044	0.96	1	0.019	2.3	3	0.013	1.7	2
Aspartic acid	0.0050	1.0	1	0.023	2.8	3	0.017	2.2	2
Methionine (oxidized)							0.0032	0.42	1
Threonine				0.0083	1.0	1	0.013	1.7	2
Serine	0.0019	0.4		0.015	1.8	2	0.021	2.8	3
Glutamic acid	0.0055	1.3	1	0.026	3.1	3	0.010	1.3	1
Proline	0.0044	1.0	2				0.0046	0.6	1
Glycine	0.0046	1.0[c]	1	0.0085	1.0[c]	1	0.0076	1.0[c]	1
Alanine				0.013	1.6	2	0.0072	0.95	1
Valine	0.0072	1.6	2	0.017	2.0	2			
Isoleucine							0.0063	0.083	1
Leucine									
Tyrosine	0.0038	0.76	1	0.014	1.7	2	0.0076	1.0	1
Phenylalanine	0.0038	0.76	1						

[a] No distinction is made between aspartic acid/asparagine and glutamic acid/glutamine in the amino acid analyses, and no correction for hydrolysis losses of threonine, serine, proline, or cysteic acid has been made here. [b] The first number in parentheses is the mobility *before* oxidation, and the second number is the mobility *after* oxidation. However, both mobilities were measured after completion of electrophoresis in the second dimension so that mobilities in the first dimension are not always identical even

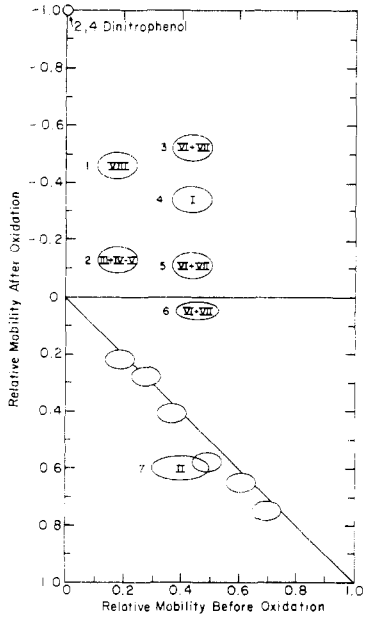


FIGURE 2: A tracing of the two-dimensional peptide map of cysteic acid peptides observed with native ribonuclease. The ellipses represent the actual dimensions (in relative mobility units) of the observed spots. The roman numeral, which appears within the ellipse, is the half-cystine residue number, according to the system of Spackman *et al.* (1960). The number which appears to the left of each off diagonal ellipse refers to the spot number of Tables III and IV. The abscissa is the relative mobility before oxidation and the ordinate is the relative mobility after oxidation. The mobility of 2,4-dinitrophenol is defined as −1.0. The peptide maps were obtained in pyridine–acetic acid–water buffer (pH 6.5).

tration over a range 5.4×10^{-6} to 5.8×10^{-5} M. The measurements were made in 0.1 M Tris-chloride buffer (pH 7.92) at 25.0° with equimolar concentrations of reduced and oxidized glutathione of 5×10^{-4} M. The average rate constants and amplitudes obtained from four experiments are included in Table II.

The kinetic data in the fluorescence measurements which were fit to eq 6 covered a time range from 30 sec to 210 min. If the rate parameters obtained are used to calculate a zero time value of the fluorescence, the calculated value is found to be less than that observed experimentally for the fully reduced enzyme (1.0 *vs.* 1.6 ± 0.2 on an arbitrary fluorescence scale). This result implies that very rapid processes may be occurring prior to the first measured point at 30 sec. Attempts to fit the zero time value by use of a three exponential equation were unsuccessful. Stopped-flow and temperature-jump fluorescence experiments did not give an adequate signal, so that this problem could not be resolved experimentally.

The problems in determining the sulfhydryl group titer during reoxidation are twofold. First, it is necessary to quench the reaction and prevent further disulfide bond formation; second, the protein must be separated from glutathione to measure the protein thiol concentration. With the *p*-hydroxymercuribenzoate method, trichloroacetic acid quenching, followed by separation of the protein, precedes reaction with the thiol reagent; whereas, with *N*-ethylmaleimide, the sulfhydryl groups are first blocked, and then the protein and glutathione are separated. When fully reduced ribonuclease is analyzed by the two methods, the results indicate that no change takes place in the sulfhydryl group titer during the work-up procedure. Both

Residues 20–29	Residues 80–103	Residues 83–109	Residues 30–45
I	VI, VII	VI, VII	II
(0.44 ± 0.02, −0.34 ± 0.05)	(0.44 ± 0.02, −0.11 ± 0.02)	(0.45 ± 0.01, 0.06 ± 0.02)	(0.40 ± 0.04, 0.60 ± 0.02)
(0.65 ± 0.11, −0.37)	(0.63, −0.21)	(0.77, 0.05)	(0.65 ± 0.11, 0.80)

Spot 4			Spot 5			Spot 6			Spot 7		
μmol	Calcd stoic.	Assumed stoic.	μmol	Calcd stoic.	Assumed stoic.	μmol	Calcd stoic.	Assumed stoic.	μmol	Calcd stoic.	Assumed stoic.
			0.017	2.1	2	0.019	2.9	3	0.060	3.0	3
						0.0064	0.97	1			
			0.0084	1.0[c]	1	0.0069	1.0	1	0.040	2.0	2
0.010	0.85	1	0.015	1.8	2	0.012	1.8	2	0.018	0.93	1
0.025	1.9	2	0.021	2.5	3	0.020	2.8	3	0.055	2.75	3
0.013	1.0	1							0.018	0.90	1
			0.025	3.0	4	0.022	3.3	3	0.039	1.95	2
0.039	3.0	3	0.026	3.1	3	0.018	2.7	2	0.018	0.90	1
0.013	1.0[c]	1	0.019	2.3	2	0.014	2.1	2			
			0.011	1.2	1				0.021	1.0	1
0.004	0.31		0.0094	1.1	1	0.0066	1.0[c]	1			
0.014	1.1	1	0.017	2.0	2	0.017	2.6	3			
						0.0063	0.96	1	0.019	0.95	1
			0.0069	0.82	1	0.012	1.8	2			
									0.020	1.0[c]	1
0.011	0.85	1	0.013	1.5	2	0.012	1.8	2			

though they originate from the same spot prior to oxidation. The three peptides found at a first dimension mobility of approximately 0.45 are postulated to consist of the peptides in spots (3, 4, 7), (4, 5, 7), and (4, 6, 7). Since spots 4 and 7 are postulated to be derived from all three peptides, the calculated first dimension mobilities for spots 4 and 7 are given as the average of those for spots 3, 5, and 6. [c] These values were assigned as the reference of 1.0.

methods give the same results for the time course of reoxidation, which is included in Figure 1. The reoxidation was carried out at 23–24° in 0.1 M Tris-chloride (pH 7.65) with equimolar concentrations of reduced and oxidized glutathione of 5×10^{-3} M and protein concentrations of $5\text{–}7 \times 10^{-5}$ M. Since the earliest measured point (1 min) is 2.70 sulfhydryl groups per molecule, the half-time for sulfhydryl group oxidation is less than 1 min. After 5 min, there are 1.2 ± 0.2 sulfhydryl groups per molecule, and this value decreases slowly with time.

To determine if the rapid decrease in sulfhydryl group titer is the result of formation of mixed disulfides with glutathione, the extent of glutathione attachment to ribonuclease (under conditions identical with those used to study the rate of disappearance of sulfhydryl groups) was measured with radioactive glutathione as described in the Experimental Section. The results obtained indicate no attachment of glutathione to native enzyme, 0.30 mol (based on the molecular weight of reduced glutathione) of reduced or oxidized glutathione attached to 1 mol of fully reduced ribonuclease at pH 3 (the zero time value for the reoxidation), 0.69 mol attached at 1 min of reoxidation, and 0.57 mol attached at 5 min of reaction. At 1 min of reoxidation, there are 2.7 disulfide bonds (on the average) formed; at 5 min of reaction, there are about 3.4 disulfide bonds formed. Even if the radioactive material is assumed to be only 65% pure, as indicated by paper chromatography, at most one mixed disulfide bond per enzyme molecule is formed between glutathione and the enzyme. Thus, after only 1 min of reoxidation, most of the disulfide bonds must be within the ribonuclease molecule.

The physical measurements in this study establish characteristics of the three-dimensional structure of the protein during reoxidation. Peptide mapping experiments were carried out to obtain information about the re-formation of particular disulfide bonds during reoxidation. For this purpose, a map of the cysteic acid peptides which result from a peptic digest of native ribonuclease was obtained, and the amino acid composition of those peptides was determined. The results of these experiments are indicated schematically in Figure 2. The dimensions of the ovals represent the approximate size of the spots; this figure represents the average of four experiments.

The results of amino acid analyses of the off diagonal spots are presented in Table III. Duplicate analyses were performed for spots 1, 2, 4, and 6. Single analyses were obtained for spots 3, 5, and 7. The error in determining amino acid composition at 0.003–0.010-μmol sample size may be ±20%, which accounts for the discrepancy between the measured and assumed stoichiometry. In any case, comparison to the known sequence of ribonuclease (Hirs *et al.*, 1960) enabled the assignments given in Table I of each spot to a specific sequence and half-cystine(s) to be made. Since the experimental conditions prevent disulfide exchange, the known pairing of half-cystines (Spackman *et al.*, 1960) applies here.

Also cited in Table III are estimates of the relative mobilities of the observed peptide, based on a theoretical relationship between mobility and the charge and mass of the peptides (Offord, 1966) (eq 7). In this equation, μ is the relative mobility, ϵ

$$\mu = k\epsilon M^{-2/3} \tag{7}$$

is the net charge, M is the mass of the peptide, and k is a proportionality constant defined from the relative mobility of known compounds. The value of ϵ is determined by summing

TABLE IV: Time Course of Peptide Mapping[a,b] during Ribonuclease Reoxidation.

Half-cystine No.	Spot No.	Time (min)							
		0	5	17	30	50	79	118	180
VIII	1						X	X	X
III + IV–V	2			X	X	X	X	X	X
VI + VII	3						X	X	X
I	4				X[c]		X	X	X
VI + VII	5						X	X	X
VI + VII	6			X	X	X	X	X	X
II	7			X	X	X	X	X	X

[a] An X indicates that the spot appears at that time during reoxidation. The peptide map for native ribonuclease indicates that prior to performic acid oxidation, one peptide is found with relative mobility 0.18 which is composed of half-cystines III, IV, V, and VIII; at a relative mobility of 0.45 three peptides of different sizes are found, but each was composed of half-cystines I, II, VI, and VII. The peptide mapping experiments cannot predict the pairing of these half-cystines in the unoxidized peptides, but the experimental conditions prohibit disulfide exchange. Therefore, the known pairing of half-cystines applies here: namely I–VI, II–VII, III–VIII, and IV–V (Spackman *et al.*, 1960). [b] The following weak intensity spots, given as time (min) (first-dimension mobility; second-dimension mobility), not found with native enzyme were also observed: 17–180 (0.43, 0.18); (0.60, 0.35); 30 (0.96, −0.33); (0.96, −0.85) both are very weak; 79 (0.70, −0.38) very weak; 118–180 (0.47, 0.32). [c] Very weak.

the charges of the individual amino acids, with cysteic acid, aspartic acid, and glutamic acid having a charge of −1, lysine and arginine +1, and histidine +¼, at pH 6.5. At this pH all other amino acids will be neutral. The value of k was determined from the measured mobilities of *N*-Dnp-glutamic acid and Methyl Green. To evaluate the validity of these calculations, which may be in error with peptides which are nonspherical or contain cysteic acid, the ratio μ(calcd)/μ(obsd) was computed. The average ratio is 1.3 ± 0.3, indicating reasonable agreement between experiment and theory.

Almost all of the spots observed during reoxidation are the same as those of the native protein, and the results are compared to those of native ribonuclease in Table IV. The significance of the results of Table IV cannot be interpreted without some estimates of the limit of detection of the ninhydrin stain. At a sample size of 4–5 mg, a spot not seen with a 1.5–3.0-mg sample size was detected. (The peptide maps used to determine the time course of reoxidation and to determine the composition of the cysteic acid peptides of native ribonuclease were obtained with a 4–5-mg sample size. The smaller sample sizes were used in initial experiments.) This spot, which contained two lysines, amounted to 3 mol % of the total recovery of cysteic acid in all peptides. This implies that a spot must contain at least 3% of the total amount of disulfide peptides in order to be detected. A spot with less lysine would give a smaller color yield and be more difficult to detect. In addition, the color constant of the particular amino acid which is the N-terminus will also influence the limit of detection, although quantitative data are not available.

Discussion

The kinetic parameters characterizing the oxidation of reduced ribonuclease in mixtures of oxidized and reduced glutathione as measured by changes in enzymatic activity, absorbance, fluorescence, and sulfhydryl titer are summarized in Table II. The dependence of the rate constant on protein concentration was examined in both the activity and fluorescence measurements, and no dependence was observed, within the experimental uncertainties. The results of Table I indicate that the rate of activity regain is not strongly dependent on glutathione concentration, except at extremely high or low ratios of reduced to oxidized glutathione. A large excess of reduced glutathione is essentially a reducing medium, while a very low ratio of reduced to oxidized glutathione apparently is ineffective in reducing incorrectly formed disulfides.

The rate constants obtained with absorbance and fluorescence measurements are essentially the same (although the glutathione concentration was tenfold lower in the fluorescence measurements to prevent quenching), and the data are quantitatively consistent with eq 6. With absorbance measurements, approximately 22% of the observed changes are associated with the faster first-order process (k_1) and 78% with the slower process (k_2). With the fluorescence measurements, the corresponding values are 40 and 60%, respectively. Both absorbance and fluorescence measurements reflect a change in the properties of the tyrosine residues as the protein refolds, but no simple relationship between the relative amplitudes of the absorbance and fluorescence experiments is required.

The single first-order rate constant characterizing the activity regain is very similar to the value of k_2 obtained by spectral methods. Apparently the rate process associated with k_1 does not appreciably alter the enzymatic activity. The apparent rate constant associated with disulfide bond formation, as determined by sulfhydryl group titrations, is considerably larger than k_1 and k_2. A very rapid change in fluorescence also may occur during the initial phase of the reoxidation process, but this change was too fast to characterize kinetically.

The oxidation rates did not vary significantly over the narrow pH range of 7.65–7.92 employed in these studies. The rate of oxidation of reduced lysozyme in glutathione solutions was also independent of pH over the range 7.4–8.2 (Saxena and Wetlaufer, 1970).

Some conclusions about the structural changes associated with the kinetic processes can be inferred from the data. Prior to the start of reoxidation, the pH of the mixture is about 3. Under these conditions the addition of equimolar reduced and oxidized glutathione does not alter the number of titratable sulfhydryl groups of the protein, indicating that either mixed disulfides between enzyme and glutathione are not formed, or the formation of disulfides is freely reversible at pH 3. This is consistent with the binding experiments indicating that very small, nonstoichiometric amounts of glutathione are strongly attached to the reduced enzyme (~0.3 mol of glutathione/mol of enzyme). Since off diagonal spots are not observed, the peptide mapping experiments also indicate that stable disulfide bonds are not formed.

The absorbance difference spectrum between native ribonuclease and reduced enzyme is essentially the same as that between native enzyme and enzyme in concentrated guanidine hydrochloride (>4 M). This indicates that all of the tyrosine residues of the reduced protein are exposed to the solvent, although tyrosine residues 97, 92, and 25 are partially buried in the interior of the protein in the native enzyme (Woody *et al.*, 1966). The fluorescent intensity of fully reduced ribonuclease is higher than that of the native protein in water, or the native protein denatured in guanidine hydrochloride. This difference could be the result of a more complete unfolding of the protein

when the constriction of the disulfide bonds is removed, but more likely it is due to the quenching of fluorescence by the disulfide bridges. Of the six tyrosine residues in ribonuclease, residues 73 and 25 are adjacent to half-cystines, and residues 92 and 97 are within close proximity of other half-cystines. Model compound measurements with L-cysteinyl-L-bistyrosine and the corresponding disulfide indicate that the cystine linkage can result in a tenfold fluorescence quenching compared to the monomer (Longworth, 1968). Flash photolysis experiments measuring the interaction of electronically excited tyrosine with the disulfide bond of sodium lipoate also indicate quenching occurs to a significant extent (Feitelson and Hayon, 1973).

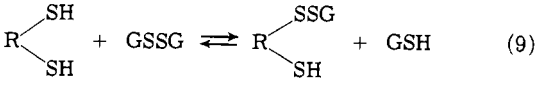
The peptide map, obtained at 5 min of reoxidation, shows no off diagonal spots. As discussed earlier the limit of detection of the ninhydrin stain requires that 2–3% of the protein be in one unique conformation in order to be detected in the peptide maps. If there were six species with differently paired cysteine residues, with each species giving seven different off diagonal spots (the number of off diagonal spots which result from a peptic digest of native ribonuclease), the resultant 42 spots would be so weak as to escape detection. Therefore six species approximates the maximum number which can be detected. A statistical analysis can be employed to test the feasibility of this estimate. The number of distinct arrangements, $N_{2n}{}^{j}$, of j disulfide bonds which can be formed, from $2n$ cysteine residues is given by the expression (Sela and Lifson, 1959; Kauzmann, 1959)

$$N_{2n}{}^{j} = (2n)!/[2^{j}(2n - 2j)!\, j!\,] \qquad (8)$$

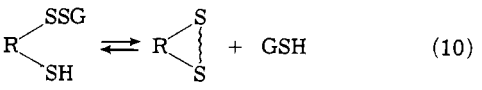
If four disulfide bonds are formed, they could be distributed over 105 statistically (if not physically) accessible structures. With three of four possible disulfide bonds formed, leaving on the average two free cysteines, 420 distinct arrangements result. This indicates that, in principle, the disulfide bonds formed during the early stages of reoxidation could be distributed over a large number (considerably greater than six) of different species. The amount of the native structure present after 5 min of reoxidation, as measured by the enzymatic activity, is about 2–3% so that detectable off diagonal spots from native enzyme would not be expected.

The best explanation of these results is that the disulfide bonds formed at 5 min, although intramolecular, are not those of the native enzyme. That is, in the initial rapid phase of the reoxidation, many conformations lead to the entrapment of incorrectly paired cysteine residues and their oxidation to disulfide bonds.

The rapid rate processes indicated by the fluorescence measurements and the time dependence of the sulfhydryl titer are consistent with the scheme shown in eq 9 and 10. In these



(9)



(10)

equations R is ribonuclease, GSSG is oxidized glutathione, GSH is reduced glutathione, and S∼∼S indicates an "incorrect," *i.e.*, nonnative conformation. Only one pair of sulfhydryl groups is represented, but the extension to eight sulfhydryl groups is obvious.

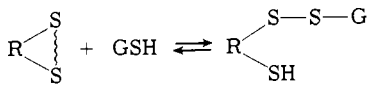
In order for this scheme to be applicable, it must be possible to form mixed disulfide bonds between cysteine and oxidized glutathione, and to exchange them in favor of intramolecular bonds, within about 1 min. Kinetic studies of the rate of formation of mixed disulfides between cystamine and glutathione, under comparable conditions to these experiments, have been carried out (Eldjarn and Pihl, 1957). The bimolecular rate constant for the formation of a mixed disulfide between ionized reduced glutathione and cystamine is 2×10^{5} M^{-1} min^{-1}. If this rate constant is assumed for the formation of a mixed disulfide between oxidized glutathione at 5×10^{-4} M and enzyme sulfhydryl groups at much lower concentrations, an apparent first-order rate constant of 100 min^{-1} is obtained. Thus, the formation of mixed disulfides within 30 sec is quite feasible.

In experiments with reduced lysozyme–cystine mixed disulfides (Bradshaw *et al.*, 1967), it was shown that eight mixed disulfides could be rearranged to form eight intramolecular bonds in 90 sec at pH 7.5 and 37°. Therefore, the mechanism proposed in reactions 9 and 10 is consistent with the observed rates of similar processes in model systems.

The rapid drop in fluorescence (occurring in less than 30 sec) could be attributed to formation of mixed disulfides, and the decay described by k_1 could be a conformational change following formation of either mixed disulfides or (more probably) intramolecular disulfides. The rate of refolding from guanidine hydrochloride solutions provides a measure of the rate of regain of structure with disulfides intact. Although the rate constant, which is dependent on the final guanidine hydrochloride concentration, appears somewhat larger than k_1, the magnitude is comparable. The rate of this chain refolding could easily be slower when the disulfides are formed incorrectly.

The first evidence for the formation of disulfide bonds seen in the peptide mapping occurs after 17 min of reoxidation (Table IV), when three off diagonal spots can be detected, which coincide with spots 2, 6, and 7 found with the native enzyme (half-cystines III + IV–V, VI + VII, and II). The corresponding pairs of these spots are lower in lysine content and therefore are not detected by ninhydrin. After 79 min, all of the spots seen with native enzyme occur, including off diagonal spots (1, 3, 5, and 4) corresponding to half-cystines VIII, VI + VII, and I which involve amino acid sequences low in lysine. The time course of the reaction as followed by peptide mapping (Table IV) unfortunately does not indicate the order of formation of disulfide bonds in the reoxidation. Instead, the time at which the off diagonal spots (identical with those found with native enzyme) appear is a combination of the sensitivity of ninhydrin to the particular amino acid sequences and the increasing concentration of native enzyme; 17 min after the initiation of the reaction, approximately 8% of the activity is restored, while at 79 min 30% of the activity has returned. These percentages presumably give a direct measure of the fraction of the enzyme in its native structure. After 180 min of reoxidation, the longest time sample examined, the peptide mapping pattern is the same as that of native enzyme in form and intensity of ninhydrin staining. The measured activity at this point is about 60% of the maximum possible value, and the spectral properties of the enzyme at this time are very nearly those of the native enzyme. Approximately one free sulfhydryl group can still be detected at long times. The only consistent evidence for incorrectly paired disulfides found in the peptide mapping experiments are two nonnative off diagonal spots which appear after 17 min of reoxidation and persist to the longest times studied. These spots apparently represent wrong pairings which do not rearrange. Since the maximum activity regained is about 90%, 10% of the enzyme might have incorrect cysteine pairings.

The slow formation of the correct disulfide bonds is characterized by the rate constant k_2 and can be schematically represented by eq 11–13.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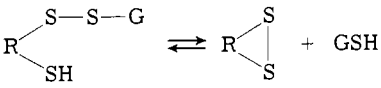
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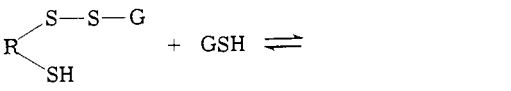

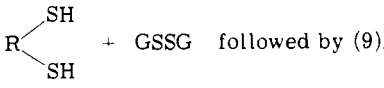
followed by (9), (10). (13)

In these equations

represents the conformation of the native enzyme. This mechanism is consistent with the observation that reduced glutathione is necessary for optimum rates of activity regain. The reduced glutathione can break incorrect disulfides and allow them to re-form correctly. As the peptide maps obtained after longer reoxidation times do not show significant concentrations of intermediates, it can only be estimated from the limitations of detection previously discussed that a minimum of six (and probably many more) wrongly paired cysteine forms of the enzyme must exist as intermediates in the reaction. Since all the intermediates are present in very low concentrations, these experiments do not establish what specific step is rate determining in reoxidation.

An important feature of these results is that although a large number of intermediates with incorrectly paired cysteines are apparently formed, 90% of the molecules find their way to the correctly folded (active) structure. Thus, the conformational space of this polypeptide appears to be readily accessible so that the enzyme converges readily to its presumably thermodynamically stable state. This provides some experimental basis for the assumption of the accessibility of conformational space made in conformational energy calculations. The approximately 10% of nonnative states formed could be metastable states, or could be intermediates trapped by trace impurities, such as heavy metals.

In terms of the proposed mechanism, the formation of a disulfide bond with correctly or incorrectly paired cysteines is energetically favored, but entropically unfavored, whereas the rearrangement of disulfide bond pairings could involve changes in both energy and entropy. The critical step in formation of the correctly paired structure could involve searching out the correct free energy minimum from a collection of wrongly paired disulfide bonded structures. As evidenced by the spectral measurements, the formation of incorrect disulfides allows only partial burying of the tyrosines, while the unique native structure allows a more complete burying.

The pattern of first-dimension peptides which results from a peptic digest of native ribonuclease can be understood in terms of the three-dimensional structure of the native enzyme (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967). Ribonuclease is roughly kidney shaped, with a cleft in the middle of one side. On one side of this depression, there are two disulfide bridges, the III–VIII and IV–V; the VI–I and II–VII bridges are on the other side. Apparently pepsin cleaves the molecule near this constriction, as there are two groups of first-dimension peptides in the electrophoretograms. One of these groups is a large peptide containing the cystines III, VIII, IV, and V; the other vertical group is composed of three peptides made up of half-cystines I, VI, II, and VII. These results, therefore, provide a direct comparison between the crystalline structure of ribonuclease and its conformation in solution.

The oxidation of reduced ribonuclease by air (Anfinsen *et al.*, 1961) and by glutathione displays significant differences. First, the sulfhydryl titer decreases slowly and uniformly in air oxidation, unlike the initial rapid, large change and subsequent slower step seen in glutathione oxidation. Second, in air oxidation, no activity returns for the first 100 min of the reaction, after which it appears to increase smoothly. The rate of regain of structure, as followed by optical rotation, parallels the oxidation of sulfhydryls in air oxidation, unlike that with glutathione where the regain of structure parallels the regain of activity only at long times. Air oxidation also is accompanied by a direct correlation between the difference absorbance at 287 nm and the change in activity.

The mechanism favored for air oxidation (Anfinsen *et al.*, 1961) is an initial random (or restricted random) pairing of half-cystines to form incorrect disulfides. Activity appears only after reshuffling and consequent formation of correct disulfides. The lag in activity regain is the result of this reshuffling. This mechanism is qualitatively similar to the mechanism for glutathione oxidation. The effect of glutathione may be (1) to hasten the rate of formation of initial (wrong) disulfides and hence compress the lag time and (2) to speed up the rearrangement process, which would also compress the lag time as well as increase the rate of reoxidation after the lag. An enzyme system capable of accelerating the rate of reoxidation of reduced ribonuclease and lysozyme has been reported (Goldberger *et al.*, 1964). However, insufficient information is available concerning the enzymatic reaction for comparison with the glutathione mediated mechanism.

The re-formation of disulfide bonds in ribonuclease apparently occurs by a different mechanism than in lysozyme (Ristow and Wetlaufer, 1973). In the latter case it was concluded that only a very limited number of structures are formed in the early stages of the reoxidation.

To summarize, the results obtained indicate that the oxidation of reduced ribonuclease proceeds in at least four stages: the formation of mixed disulfides between enzyme and glutathione, formation of incorrect intramolecular disulfide pairs, a conformational transition (characterized by k_1), and finally a reshuffling of the disulfide pairings to give the native structure (characterized by k_2). A large number of intermediates with incorrectly paired cysteines are formed which are thermodynamically driven to the native structure.

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Fluorotyrosine Alkaline Phosphatase. ^{19}F Nuclear Magnetic Resonance Relaxation Times and Molecular Motion of the Individual Fluorotyrosines†

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ABSTRACT: Alkaline phosphatase from *Escherichia coli* has been labeled *in vivo* with *m*-fluorotyrosine and the ^{19}F nuclear magnetic resonance (nmr) spectrum of the fully active labeled protein shows 11 resolvable resonances corresponding to the 11 known tyrosines per subunit. Nuclear spin relaxation times T_1 and T_2 have been determined for each ^{19}F resonance. Consideration of the theory of dipole-dipole relaxation between unlike spins (^1H and ^{19}F) results in the following conclusions. First, the relaxation times are insensitive to internal rotation about the C_β-aromatic ring bond. Secondly, the data require that motion about the C_α-C_β bond have a correlation time of $\gtrsim 10^{-6}$ sec; hence, such motion does not contribute significantly to relaxation. All of the relaxation data are well represented by a model which assumes (1) isotropic motion of the protein as a whole with a rotational correlation time $\tau_c \simeq 70$ nsec and (2) a varying degree of "intermolecular" contribution to the ^{19}F relaxation in tyrosine residues by protons on nearby residues. Finally, the "intermolecular" relaxation exhibited a strong correlation with the ^{19}F chemical shift; the contribution of "intermolecular" relaxation was roughly proportional to the shift of a tyrosine from the position of the denatured protein resonance. Thus, ^{19}F nmr is a very useful tool for studying the general tertiary or quaternary structure of a protein, its motional properties, and differences in the local environments of particular residues.

The alkaline phosphomonoesterase (EC 3.1.3.1) from *Escherichia coli* is a metalloprotein consisting of two identical subunits (mol wt 86,000/dimer) and requires at least two tightly bound zinc atoms for full activity (Applebury and Coleman, 1969; Reynolds and Schlesinger, 1969; Csopak *et al.,* 1972). This enzyme catalyzes the hydrolysis of a wide variety of phosphate esters with very little specificity for substrate. The catalytic mechanism of alkaline phosphatase has been a subject of considerable interest in recent years and it appears that at alkaline pH a slow enzyme conformational change is the rate-limiting step and thus responsible for the uniform rates of substrate hydrolysis (Halford, 1972). Controversial questions of stoichiometry and subunit interaction are also of importance in understanding the mechanism of alkaline phosphatase (Lazdunski *et al.,* 1971; Bloch and Schlesinger, 1973).

In order to obtain additional specific information concerning the structure-function relationships for alkaline phosphatase, we have undertaken a study of the protein structure using a

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