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Why Does Ribonuclease Irreversibly Inactivate at High Temperatures?†

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ABSTRACT: The mechanism of irreversible thermoinactivation of bovine pancreatic ribonuclease A in the pH range relevant to enzymatic catalysis has been elucidated. At 90 °C and pH 4, the enzyme inactivation is caused by hydrolysis of peptide bonds at aspartic acid residues (the main process) and deamidation of asparagine and/or glutamine residues. At 90 °C and neutral pH (pH 6 and 8), the enzyme inactivation is caused by a combination of disulfide interchange (the main process), β-elimination of cystine residues, and deamidation of asparagine and/or glutamine residues. These four processes appear to demarcate the upper limit of thermostability of enzymes.

Enzymes (proteins) are exposed to high temperatures in a variety of real-life situations including thermophilic microorganisms living in hot springs and hydrothermal vents, industrial biochemical reactors, food preparation, heat sterilization of fermentation media, etc. Therefore, elucidation of molecular events taking place in proteins upon heating is of fundamental significance for both pure and applied biochemistry. Knowledge of why and how proteins lose their biological activity is crucial for understanding thermophilic behavior (Zuber, 1976; Friedman, 1978) and also should demarcate the upper limit of thermostability of proteins and hence of life (Brock, 1985). Continuing controversy over whether bacteria can live at 250 °C (Baross & Deming, 1983, 1984; Walsby, 1983; Trent et al., 1984; White, 1984) further underscores the timeliness and importance of such research. In addition, a mechanistic understanding of enzyme stability and inactivation is required for the emerging area of stabilization of enzymes by protein engineering (Ulmer, 1983; Perry & Wetzel, 1984; Estell et al., 1985).

Upon heating in aqueous solutions, the following processes occur in enzymes (Klibanov, 1983): First, the enzyme molecule partially unfolds due to heat-induced disruption of the delicate balance of various noncovalent interactions (Schulz & Schirmer, 1979; Creighton, 1983) that maintain the native conformation at ambient temperature. This process, which results in enzyme inactivation, is completely *reversible*: the enzymatic activity is fully regained if the enzyme solution is promptly cooled down. However, upon prolonged heating, a gradually decreasing fraction of the enzymatic activity returns after cooling, indicating that another, *irreversible* process also takes place. Thus, the overall phenomenon can be depicted by the classical scheme of Lumry and Eyring (1954):

$$N \rightleftharpoons U \rightarrow I \qquad (1)$$

where N is the native, catalytically active enzyme, U is the reversibly unfolded, catalytically inactive enzyme, and I is the irreversibly inactivated enzyme.

A great deal is known about the first step in eq 1 (Kauzmann, 1959; Tanford, 1968; Lapanje, 1978; Privalov, 1979; Jaenicke, 1981; Pfeil, 1981) which is often called thermal denaturation or melting of proteins. The origin and mechanisms of this process are conceptually straightforward and well understood. On the other hand, the nature of the second, irreversible step in eq 1 has remained somewhat mysterious for decades. This process does not render itself to well-defined thermodynamic studies and therefore is usually viewed as simply a nuisance by enzymologists and protein chemists, and yet, this process is the cause of enzyme deterioration in commercial biocatalytic reactors and hence plagues further progress of enzyme technology (Klibanov, 1983). Irreversible thermoinactivation of enzymes has been ascribed to a variety of diverse mechanisms (Baldwin, 1975; Whitaker, 1972; Tombs, 1985), of which none is generally applicable and many have not withstood experimental tests (Klibanov, 1983).

Recently, we have succeeded in elucidating the mechanisms of irreversible thermal inactivation of hen egg white lysozyme (Ahern & Klibanov, 1985). The processes leading to thermoinactivation were found to be deamidation of Asn/Gln residues, hydrolysis of peptide bonds at Asp residues, destruction of disulfide bonds, and formation of incorrectly folded and kinetically trapped structures; their relative contributions depended on the pH. To test the generality of the uncovered mechanisms, in the present work we have investigated irreversible thermoinactivation of another, unrelated enzyme, bovine pancreatic ribonuclease A. Ribonuclease was chosen as a model because it is a relatively small, monomeric enzyme, containing no non-protein components, whose structure is well established (Richards & Wyckoff, 1971; Blackburn & Moore, 1982) and whose reversible thermal denaturation (Hermans & Scheraga, 1961; Brandts, 1965) and conformational dy-

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namics have been studied extensively (Tanford, 1968; Lapanje, 1978; Anfinsen & Scheraga, 1975). In the temperature range from 80 to 100 °C, bovine pancreatic ribonuclease undergoes irrerversible thermoinactivation, the mechanism of which heretofore remained obscure (Zale & Klibanov, 1983). This paper provides the answer to the title question.

Experimental Procedures

Materials. Bovine pancreatic ribonuclease A (EC 3.1.27.5) was purchased from Sigma Chemical Co. (St. Louis, MO) as a powder with a specific activity of 95–100 Kunitz units/mg of protein (type XII-A). The enzyme was found to be homogeneous by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) (Laemmli, 1970), by nonequilibrium isoelectric focusing (O'Farrell et al., 1977), and by gel permeation chromatography on Sephadex G-75. The amino acid composition of the enzyme, determined by us using high-pressure liquid chromatography (HPLC), was in good agreement with the literature data (Smyth et al., 1963).

Bovine liver glutamate dehydrogenase (EC 1.4.1.3), rabbit muscle L-lactate dehydrogenase (EC 1.1.1.27), bakers' yeast carboxypeptidase Y (EC 3.4.16.1), *S*-acetylcoenzyme A synthase (EC 6.2.1.1), porcine heart citrate synthase (EC 4.1.3.7), malate dehydrogenase (EC 1.1.1.37), and torula yeast ribonucleic acid (RNA) were also obtained from Sigma. All other chemicals used were of the highest purity commercially available.

Ribonuclease Assay. The enzymatic activity of ribonuclease was measured spectrophotometrically by using torula yeast RNA as a substrate, in accordance with the procedure outlined by Kunitz (1946). Protein concentrations were measured by Lowry's method (Lowry et al., 1951), unless stated otherwise.

Kinetics of Irreversible Thermoinactivation of Ribonuclease. The time course of irreversible thermoinactivation of ribonuclease was measured by incubating aqueous solutions of the enzyme (generally at a concentration of 0.67 mg/mL in a buffered system that had been adjusted to the desired pH at room temperature) at 90 °C in a thermostatically controlled glycerol bath and periodically removing samples and assaying them for enzymatic activity at 25 °C. First-order rate constants of irreversible thermoinactivation were obtained by linear regression in semilogarithmic coordinates.

Reactivation of "Irreversibly" Thermoinactivated Ribonuclease. Thermoinactivated ribonuclease was reactivated in accordance with the general approach of Klibanov and Mozhaev (1978). Reduction was carried out by using 0.07 M dithiothreitol in a 6 M aqueous solution of guanidine hydrochloride according to the method of Bibring and Baxandall (1978). Following desalting on Sephadex G-25, the reduced enzyme was allowed to reoxidize for 18 h in the presence of a mixture of 0.5 mM each of reduced and oxidized glutathione and 1 mM ethylenediaminetetraacetic acid (EDTA) in 1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl) buffer at pH 8.0 (Chavez & Scheraga, 1980). The specific activity of the reactivated preparation was measured by using the assay outlined above.

SDS–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of SDS was carried out according to the method of Laemmli (1970). Samples were subjected to electrophoresis at a constant current of 20 mA on 15% polyacrylamide gels. Protein was stained with Coomassie Blue R-250.

Reduction and Carboxymethylation of Ribonuclease. Samples of native and thermoinactivated ribonuclease were reduced and carboxymethylated prior to acid hydrolysis for amino acid analysis, hydrazinolysis, digestion with carboxypeptidase Y, dansyl chloride end group analysis, or free amino group titration with trinitrobenzenesulfonic acid. The carboxymethylation method employed was based on that described by Bibring and Baxandall (1978): To a solution of 1 mg of native or thermoinactivated ribonuclease in 4.8 mL of 0.5 M Tris·HCl buffer containing 6 M guanidine hydrochloride and 0.3% EDTA (pH 8.6) was added 50 mg of dithiothreitol (to give a final concentration of 0.07 M) and the mixture was incubated overnight under an argon atmosphere. Addition of 0.6 mL of 1 M iodoacetic acid in 1 N NaOH, followed by a 20-min incubation under argon and desalting on Sephadex G-10, yielded S-carboxymethylated ribonuclease. Amino acid analysis revealed that all eight sulfhydryls of reduced ribonuclease were modified by this procedure.

Determination of Amino Acid Composition. Samples of native and thermoinactivated ribonuclease that had been reduced and carboxymethylated were degassed and hydrolyzed in 6 N HCl (Pierce) at 110 °C for 20 h. The amino acid compositions of the hydrolysates were determined by using reverse-phase HPLC with precolumn derivatization with *o*-phthalaldehyde (Fernstrom & Fernstrom, 1981). Since this method does not separate glycine and threonine and cannot detect proline or lysinoalanine, reduced and carboxymethylated samples of native ribonuclease and ribonuclease thermoinactivated both at pH 4 and 8 were also examined by amino acid analysis using a conventional amino acid analyzer (Durrum D-500) with postcolumn derivatization with ninhydrin (Hare, 1975).

Determination of Free Sulfhydryl Groups. The titer of sulfhydryl groups in thermoinactivated samples of ribonuclease or in samples of the reduced enzyme was determined by using Ellman's reagent (Ellman, 1959) according to the procedure outlined by Taniuchi (1970). The measured sulfhydryl content of reduced ribonuclease was in good agreement with the expected value of 8.0 SH groups per molecule. The assay was not affected by small concentrations of cupric chloride (corresponding to 10 μM in the sample).

Determination of Hydrosulfide. Hydrosulfide (HS^-) determination was based on the method described by Rabinowitz (1978). One-milliliter samples were removed from the solution of the enzyme that was undergoing thermoinactivation and immediately added to 0.65 mL of zinc acetate solution (2% in water) on ice. Fifty microliters of 12% NaOH solution was added, and the samples were removed from the ice bath. Dimethylphenylenediamine dihydrochloride (0.25 mL of a 0.1% solution in 5.5 N HCl) and $FeCl_3$ (50 μL, 23 mM in 1.2 N HCl) were added, and the tubes were allowed to stand at room temperature for 30 min. The absorbance of the samples at 670 nm was measured and compared to a standard curve prepared by using known concentrations of hydrosulfide. The assay was not affected by the presence of small concentrations of cupric chloride (corresponding to 10 μM in the sample).

Determination of Dehydroalanine. The time course of appearance of dehydroalanine residues during thermoinactivation of ribonuclease was measured by acid hydrolysis and amino acid analysis of samples that had been treated with 0.2 M sodium sulfite, which reacts with dehydroalanine residues to yield cysteic acid residues (Strumeyer et al., 1963).

Determination of Ammonia. The time course of evolution of ammonia during thermoinactivation of ribonuclease was determined by incubating samples of the enzyme in sealed ampules at 90 °C for various periods of time. All of the ampules were then opened, and the amount of dissolved ammonia was measured enzymatically with glutamate dehydrogenase (Kun & Kearney, 1974).

Isoelectric Focusing. The time course of appearance of deamidated forms of ribonuclease was determined by using nonequilibrium isoelectric focusing according to the method of O'Farrell et al. (1977). Samples containing approximately 20 μg of protein were focused for 2.5 h at 200 V in a 1.5-mm-thick 4% polyacrylamide gel containing 8 M urea and 5% Ampholine 7-9 (LKB) that had been prefocused for 1 h at the same voltage. Gels were stained with 0.25% Coomassie Blue in methanol/acetic acid/water (50:7:43) and then destained with methanol/acetic acid/water (40:10:50). The percentages of native and mono-, di-, tri-, and tetradeamidated ribonucleases in each sample were determined by using gel densitometry (Bio-Rad Model 1650).

To determine the effect of deamidation on the specific activity of ribonuclease, isoelectric focusing was carried out on a preparative scale. Samples containing 1–1.5 mg of ribonuclease that had been heated for 2 h at pH 6 and then unscrambled by reduction and reoxidation of S–S bonds were applied to the gel in 6-cm-wide wells. After being focused, vertical slices of the gel were removed and stained with Coomassie Blue in order to locate the bands corresponding to the deamidated forms of the enzyme. Then, the gel was reassembled, and the unstained portions of the gel that contained protein were cut out, extracted overnight with 1.5 mL of 1 M acetate buffer (pH 5.0), and desalted on Sephadex G-25. Samples containing native and mono-, di-, and trideamidated ribonucleases were obtained in this manner and were found to be homogeneous using analytical isoelectric focusing.

Determination of Free Amino Groups. Free amino groups in ribonuclease were assayed spectrophotometrically by using trinitrobenzenesulfonic acid in accordance with the method of Fields (1971). A calibration curve was prepared by using *N*-acetyl-L-lysine. Ribonuclease that had been reduced and carboxymethylated (see below) was found to have a titer of 11.6 ± 0.3 free amino groups per molecule, in reasonable agreement with the expected value of 11.0 (Smyth et al., 1963).

Acetylation of Ribonuclease. Ribonuclease was acetylated in the presence of the ligand sodium pyrophosphate using the same procedure as that described for succinylation of ribonuclease (Giese & Vallee, 1972) except that acetic anhydride was substituted for succinic anhydride.

Determination of Acetate. Acetate was assayed by using the enzymatic procedure of Beutler (1984).

Hydrazinolysis. Hydrazinolysis of ribonuclease was performed by using the resin-catalyzed method of Braun and Schroeder (1967). S-Carboxymethylated samples (ca. 10 nmol) of native and thermoinactivated ribonuclease were placed in 1-mL ampules that contained 5 mg of Amberlite CG 50. Anhydrous hydrazine (0.4 mL) was added, and the ampules were sealed under argon. The samples were incubated at 80 °C for 24 h and then opened, lyophilized, and resuspended in 0.2 mL of aqueous pyridinium acetate buffer (pH 5.6) containing 20 μM α-aminobutyric acid (an internal standard). Amino acid hydrazides were removed by passing the samples through 0.4 × 2 cm Amberlite CG 50 columns that had been equilibrated with the same buffer. The samples were then lyophilized, dissolved in water, and analyzed for free amino acids by HPLC. Hydrazinolysis of native S-carboxymethylated ribonuclease indicated that valine was the C-terminal amino acid, as expected from the known sequence of the enzyme (Smyth et al., 1963).

Digestion with Carboxypeptidase Y. Carboxypeptidase Y digestion of carboxymethylated samples of native and thermoinactivated ribonuclease was based on the method of Hayashi (1977). Digestion was carried out in 200 μL of 50 mM sodium acetate buffer, pH 5.3, containing approximately 40 nmol of the carboxymethylated sample, 0.4 nmol of carboxypeptidase Y, and 0.1 mM α-aminobutyric acid (an internal standard). Aliquots were removed, diluted into ice-cold water, and immediately frozen on a dry ice/acetone bath. Samples were stored in the freezer prior to HPLC determination of liberated amino acids. Carboxypeptidase Y digestion of native, carboxymethylated ribonuclease yielded results consistent with the known sequence of the enzyme (Smyth et al., 1963).

Dansyl Chloride End Group Determination. N-Terminal amino acid residues of native and thermoinactivated ribonuclease were determined by using the microdansylation method of Fleischman (1973). Carboxymethylated samples (~0.4 nmol) were incubated with dansyl chloride in the presence of SDS at 45 °C for 30 min. The dansylated proteins were then hydrolyzed in 6 N HCl (110 °C, 4 h) and analyzed by two-dimensional thin-layer chromatography on polyamide plates. Spots were visualized by using UV light. Standard mixtures of dansylated amino acids were also spotted on the plates in order to facilitate identification of N-terminal residues. Spots corresponding to "bis-lysine" (lysine dansylated in both the α- and ε-amino groups) were present on all plates, consistent with the presence of a lysine residue at the amino terminus of ribonuclease (Smyth et al., 1963).

Results and Discussion

The objective of this study was the determination of the exact mechanism of irreversible thermoinactivation of bovine pancreatic ribonuclease A. Our aim was to identify the process(es) actually causing the inactivation, as opposed to those occurring simultaneously but having nothing to do with it. Nearly all thermoinactivation experiments were carried out at 90 °C, where the enzyme inactivates at a conveniently measurable pace. The enzyme thermoinactivation was investigated in the pH range from 4 to 8, which covers the range relevant to enzymatic catalysis, and yet is sufficiently broad so that different mechanisms may contribute to irreversible thermoinactivation.

When an aqueous solution of ribonuclease at pH 5 is heated above 80 °C, the enzyme immediately loses its catalytic activity (Kalnitsky & Resnick, 1959). If the enzyme solution is quickly cooled to 25 °C, the enzymatic activity is fully regained. This process is the well-known *reversible* thermal denaturation of ribonuclease (Hermans & Scheraga, 1961; Brandts, 1965). At 90 °C, ribonuclease is completely reversibly thermodenatured throughout the entire pH range from 4 to 8 (Zale & Klibanov, 1983). If heating at 90 °C was continued, then only a fraction of the initial catalytic activity of ribonuclease could be recovered upon cooling. As described in the introduction, this is symptomatic of *irreversible* enzyme thermoinactivation. The observed inactivation was for all practical purposes irreversible: 3-day incubation of thermoinactivated ribonuclease at 25 °C resulted in no appreciable return of enzymatic activity.

Irreversible thermal inactivation of enzymes is often attributed to various polymolecular processes, e.g., aggregation (Joly, 1965; Baldwin, 1975). We have previously shown (Zale & Klibanov, 1983) that this is not the case for ribonuclease at 90 °C between pH 4 and 8 (at least in the concentration range from 0.05 to 0.67 mg/mL, in which all experiments performed in this work were carried out). This conclusion, supported by both kinetic data and the absence of water-insoluble aggregates (Zale & Klibanov, 1983), was further confirmed by our experiments employing gel permeation

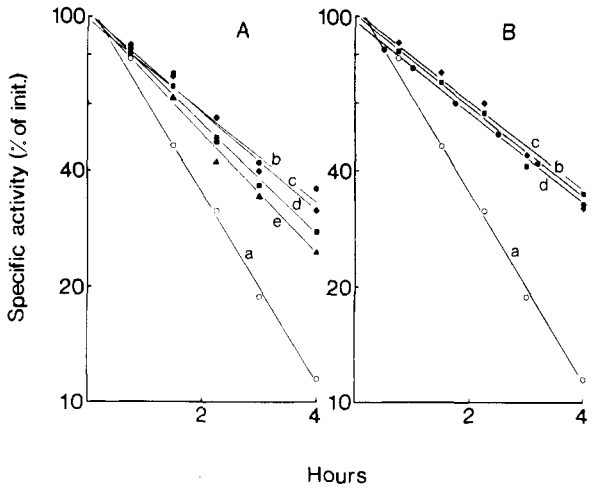


FIGURE 1: Irreversible thermoinactivation of ribonuclease at pH 6 and 90 °C. (A) Reactivation of irreversibly thermoinactivated ribonuclease. The specific activities of thermoinactivated samples were determined (curve a) (○) and then remeasured after reactivation (i.e., conversion of I_2 to E; see eq 2) under the following conditions: (b) (●) reduction followed by reoxidation at a protein concentration of 4 μM, as described under Experimental Procedures; (c) (♦) incubation with 1 mM cysteine in 1 M Tris·HCl/1 mM EDTA (pH 8.0) at a protein concentration of 20 μM; (d) (■) incubation with 0.5 mM each of reduced and oxidized glutathiones in 1 M Tris·HCl/1 mM EDTA (pH 8.0) at a protein concentration of 20 μM; (e) (▲) incubation with 1.4 mM 2-mercaptoethanol and 2.5 mM 2-hydroxyethyl disulfide in 0.1 M NH_4HCO_3 (pH 7.8) at a protein concentration of 4.0 μM. Thermoinactivation was carried out at a protein concentration of 40 μM in 0.01 M phosphate buffer containing 0.15 M KCl. (B) Effect of thiol inhibitors on the time course of irreversible thermoinactivation. (a) (○) No additive; (b) (■) 1 mM *N*-ethylmaleimide; (c) (♦) 1 mM *p*-(chloromercuri)benzoate; (d) (●) 10 μM $CuCl_2$. Thermoinactivation conditions were the same as in (A).

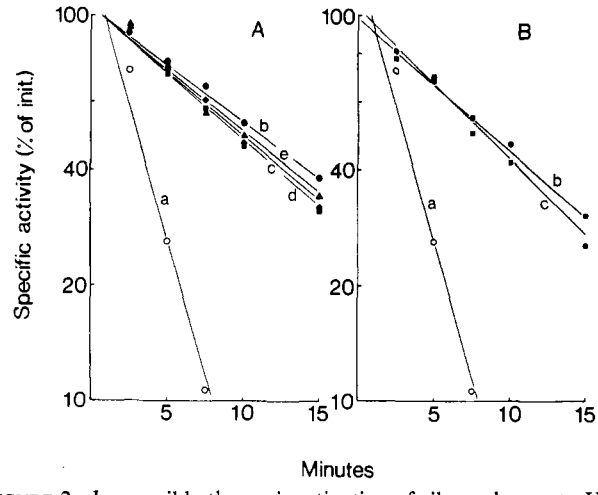


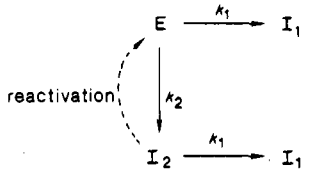
FIGURE 2: Irreversible thermoinactivation of ribonuclease at pH 8 and 90 °C. (A) Reactivation of irreversibly thermoinactivated ribonuclease. The specific activities of thermoinactivated samples were determined (curve a) (○) and then remeasured after reactivation (i.e., conversion of I_2 to E; see eq 2) under the following conditions: (b) (●) reduction followed by reoxidation, or incubation with (c) (♦) 1 mM cysteine, (d) (■) 0.5 mM each of reduced and oxidized glutathiones, or (e) (▲) 1.4 mM 2-mercaptoethanol and 2.5 mM 2-hydroxyethyl disulfide. Reactivation conditions are detailed in the legend to Figure 1. Thermoinactivation was carried out at a protein concentration of 40 μM in 0.01 M phosphate buffer containing 0.15 M KCl. (B) Effect of thiol inhibitors on the time course of irreversible thermoinactivation. (a) (○) No additive; (b) (■) 5 mM *N*-ethylmaleimide; (c) (●) 10 μM $CuCl_2$. Thermoinactivation conditions were the same as in (A).

chromatography and polyacrylamide gel electrophoresis. Therefore, at 90 °C in the pH range from 4 to 8, ribonuclease undergoes a monomolecular, irreversible thermoinactivation.

Irreversible Thermal Inactivation of Ribonuclease at Neutral pH. Experiments were conducted at both pH 6 and pH 8 in order to span the neutral pH range and to determine the pH dependence of the processes involved.

(A) Reactivation of Irreversibly Thermoinactivated Ribonuclease. The first line of investigation was to establish whether "irreversible" thermoinactivation of ribonuclease at neutral pH was due to alteration(s) in the enzyme's primary structure. The approach employed was to attempt to reactivate the enzyme (Klibanov & Mozhaev, 1978) by complete reduction in the presence of a denaturant, followed by reoxidation of S–S bonds in the absence of denaturant, i.e., under native conditions. It is expected that this procedure will overcome any kinetic barriers [e.g., incorrect disulfide bond pairings (Foster, 1960) or metastability of incorrect structures (Klibanov, 1983)] that prevent an inactivated enzyme molecule with an unaltered primary structure from reassuming its native catalytically active and thermodynamically most stable conformation upon cooling. On the other hand, those barriers resulting from covalent alterations of the enzyme's primary structure should not be surmounted.

Samples of ribonuclease that had been heated for various lengths of time at pH 6 or 8 and 90 °C were individually reduced (Bibring & Baxandall, 1978), desalted, and reoxidized (Chavez & Scheraga, 1980). The specific activity of each sample was determined and compared with that of the sample prior to reduction and refolding. It was found (Figures 1A and 2A) that a significant portion of the activity that had been lost during thermoinactivation at either pH could be recovered. To assess quantitatively the contribution of processes that can be reversed by reduction and refolding toward the overall thermoinactivation of the enzyme, a kinetic scheme (eq 2) was



(2)

employed in which the enzyme (E) can undergo irreversible thermoinactivation via either of two types of processes: (i) those that result in a thermoinactivated enzyme with an altered primary structure (I_1), governed by rate constant k_1, and (ii) those that result in a thermoinactivated enzyme with an unaltered primary structure (I_2), governed by rate constant k_2. It is assumed that thermoinactivated enzyme molecules with intact primary structures can be reactivated and that these molecules undergo changes in the primary structure at rates similar to that of the native enzyme. The purpose of this analysis is to devise an approach toward obtaining values for rate constants k_1 and k_2, as well as for the rate constant of the overall observed thermoinactivation, k_{obsd}.

The rate of disappearance of native ribonuclease is

$$-\frac{d[E]}{dt} = k_1[E] + k_2[E] = (k_1 + k_2)[E] = k_{obsd}[E] \quad (3)$$

Since enzymatic activity is directly proportional to [E]

$$\frac{d\ \ln(\text{activity})}{dt} = -(k_1 + k_2) = -k_{obsd} \quad (4)$$

Therefore, the value of k_{obsd} can be obtained by plotting activity vs. thermoinactivation time in semilogarithmic coordinates and measuring the slope. This is the general method for measurement of rate constants of first-order decay processes.

Determination of the rate of disappearance of [E] + [I_2] will provide the value of k_1, the rate constant of thermo-

inactivation due to processes that involve changes in primary structure because

$$-\frac{d([E] + [I_2])}{dt} = k_1([E] + [I_2]) \quad (5)$$

Since reactivation results in the conversion of I_2 to E, the activity of reactivated samples is directly proportional to [E] + [I_2]. Therefore, after each sample is reactivated

$$-\frac{d \ln(\text{activity}_{\text{react}})}{dt} = k_1 \quad (6)$$

and hence the value of k_1 can be obtained by plotting the activity of reactivated samples vs. thermoinactivation time in semilogarithmic coordinates and determining the slope.

The value of k_2, the rate constant of thermoinactivation due to processes that do not involve changes in the enzyme's primary structure, is obtained by difference, since $k_{obsd} = k_1 + k_2$. Therefore, the relative contributions of processes that do and do not result in thermoinactivated enzyme molecules with altered primary structures toward the overall thermoinactivation can be evaluated by comparison of the time courses of thermoinactivation determined before and after the samples have been reactivated.

This kinetic analysis was applied to thermoinactivation of ribonuclease at pH 6 (Figure 1A) and 8 (Figure 2A). For thermoinactivation at pH 6, the measured values for k_{obsd} and k_1 are 0.56 h^{-1} and 0.25 h^{-1}, respectively, and the calculated value for k_2 is therefore 0.31 h^{-1} (i.e., 55% of the overall rate constant). At pH 8, values of 23.4 and 4.0 h^{-1} were obtained for k_{obsd} and k_1, respectively, and hence 19.4 h^{-1} for k_2. This represents a contribution of 83% toward the overall process. Thus, thermoinactivation of ribonuclease at neutral pHs is mainly due to a process that can be reversed by reduction/reoxidation of S–S bonds.

A natural candidate for such a process is the thermally induced interchange of disulfide bonds, which will result in enzyme molecules with incorrect S–S bond pairings, an altered three-dimensional structure, and hence no catalytic activity. Ribonuclease with scrambled disulfide bonds (Haber & Anfinsen, 1962; Creighton, 1979) can be rearranged to yield native, catalytically active ribonuclease by incubating the protein with small amounts of thiols, such as mercaptoethanol or cysteine, which, by catalyzing the interchange of S–S bonds, facilitate the reassumption of the correct disulfide pairings (Haber & Anfinsen, 1962; Galat et al., 1981).

On the basis of these findings, it was expected that if thermoinactivation of ribonuclease occurs due to disulfide interchange, it should be possible to reactivate the scrambled enzyme simply by treating it with small amounts of thiols. This was tested by incubating samples of ribonuclease that had been thermoinactivated for varying lengths of time at either pH 6 or pH 8 with different thiols or mixtures of reduced and oxidized sulfhydryl reagents for 24 h at room temperature. These results are shown in Figures 1A and 2A for samples thermoinactivated at pH 6 and 8, respectively. In all cases, treatment with thiols resulted in substantial reactivation of the enzyme. Strikingly, at either pH, the extent of reactivation was essentially the same as that observed when the thermoinactivated enzyme was reactivated by reduction/oxidation. These results are strongly indicative that the major process contributing to ribonuclease thermoinactivation at neutral pH results in enzyme molecules with scrambled S–S bonds.

(B) Heat-Induced Disulfide Bond Interchange in Ribonuclease. The disulfide exchange reaction has been studied extensively (Cecil & McPhee, 1959; Lumper & Zahn, 1965). At neutral pH, the reaction requires the presence of catalytic amounts of thiols which promote the interchange by nucleophilic attack on the sulfur atom of a disulfide (Torchinsky, 1981). Using model peptides, Ryle and Sanger (1955) found that reagents that block SH groups [such as *p*-(chloromercuri)benzoate and *N*-ethylmaleimide] inhibit the disulfide exchange reaction and that the reaction is accelerated by addition of thiols.

If ribonuclease undergoes irreversible thermoinactivation in accordance with this type of mechanism, the analogous behavior can be expected. This prediction was experimentally tested first by investigating the effects of (i) *N*-ethylmaleimide and *p*-(chloromercuri)benzoate, which react rapidly and specifically with thiols, and (ii) copper ion, which catalyzes the spontaneous air oxidation of thiols (Torchinsky, 1981), on the time course of thermoinactivation of ribonuclease at pH 6. The results of this experiment are shown in Figure 1B, curves b–d. The time course of thermoinactivation without additives (curve a) is also shown. It can be seen that addition of thiol inhibitors results in significant stabilization of the enzyme. Furthermore, the time course of inactivation in the presence of each of the thiol inhibitors coincides closely with the time course of ribonuclease thermoinactivation after reduction and refolding (Figure 1A, curve b). The same approach was then applied to thermoinactivation at pH 8 where it was found that *N*-ethylmaleimide and $CuCl_2$ (Figure 2, curves b and c, respectively) each stabilized the enzyme by a factor of 5, which is nearly the maximum stabilization expected. As with thermoinactivation at pH 6, the kinetics of inactivation under conditions where thiols are excluded are the same as the kinetics of inactivation after the conformational process is reversed. Samples of ribonuclease that had been inactivated in the presence of either *N*-ethylmaleimide or $CuCl_2$ could not be reactivated by reduction and reoxidation conducted in the same manner as described previously. Therefore, the process that is reversed by reduction and reoxidation or by incubation with thiols can also be prevented by eliminating thiols from the system during thermoinactivation. These findings strongly suggest that this process indeed is the thiol-catalyzed disulfide exchange reaction.

Another avenue for evaluation of the disulfide exchange hypothesis is to test the effect of added thiols, which are known to accelerate the interchange reaction (Ryle & Sanger, 1955), on the kinetics of thermoinactivation of ribonuclease. Addition of 20 μM cysteine in the presence of 1 mM EDTA, to prevent autoxidation, resulted in a great increase in the rate of thermoinactivation of the enzyme. Incubation for 1 h at pH 6 and 90 °C resulted in loss of 95% of enzymatic activity, while only 40% inactivation was observed in the absence of the exogenous thiol. As one would expect, ribonuclease inactivated in the presence of cysteine could be reactivated by reduction, followed by reoxidation and refolding, to yield the same specific activity as the corresponding enzyme that had been inactivated without cysteine and then reactivated in the same manner. Therefore, addition of cysteine accelerates the process that yields thermoinactivated enzyme that can be reactivated but has no effect on the processes that do not.

The findings presented so far can be summarized as follows: The major process contributing to thermoinactivation of ribonuclease at both pH 6 and pH 8 can be reversed either by reducing the S–S bonds of the protein molecule and then allowing them to re-form under native conditions or by incubating the inactivated enzyme with small amounts of thiols. The process can be eliminated by carrying out thermoinactivation in the presence of thiol inhibitors and can be accel-

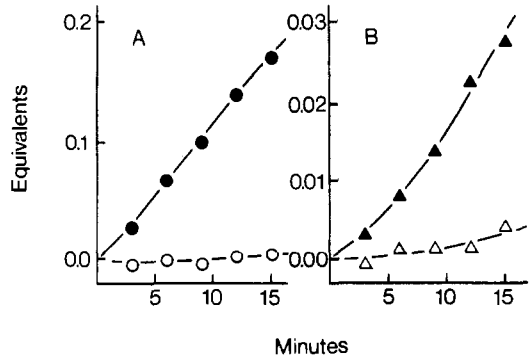


FIGURE 3: Production of thiols during thermoinactivation of ribonuclease at pH 8 and 90 °C. (A) Time course of thiol concentration in the absence (●) and presence (○) of 10 μM $CuCl_2$. Samples were assayed for sulfhydryl groups using Ellman's reagent (Taniuchi, 1970). (B) Time course of hydrosulfide concentration in the absence (▲) and presence (△) of 10 μM $CuCl_2$. Hydrosulfide was determined by using the methylene blue assay of Rabinowitz (1978). Thermoinactivation conditions were the same as in Figure 2.

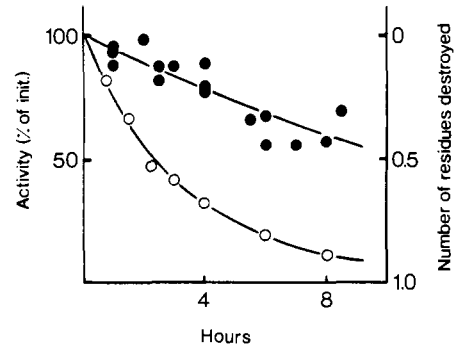


FIGURE 4: Destruction of cystine residues of ribonuclease at pH 6 and 90 °C. (●) Number of cystines destroyed; (○) specific activity of thermoinactivated samples after unscrambling by reduction/reoxidation. Cystines were determined as *S*-(carboxymethyl)cysteine as described under Experimental Procedures. Thermoinactivation conditions were the same as in Figure 1.

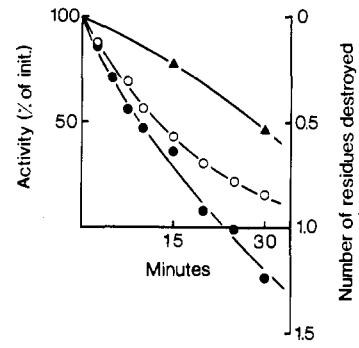


FIGURE 5: Irreversible thermoinactivation of ribonuclease at pH 8 and 90 °C due to changes in the primary structure. (○) Specific activity of thermoinactivated samples after unscrambling by reduction/reoxidation; (●) number of cystine residues destroyed; (▲) number of lysine residues destroyed. Cystines were determined as *S*-(carboxymethyl)cysteine. Thermoinactivation conditions were the same as in Figure 2.

erated by addition of thiols. These results constitute compelling evidence that the process in question indeed is the thiol-catalyzed disulfide interchange reaction.

Direct evidence that disulfide exchange occurs under the conditions used for thermoinactivation was obtained by heating the enzyme in the presence of *N*,*N*′-bis(2,4-dinitrophenyl)-L-cystine, which resulted in incorporation of the 2,4-dinitrophenyl chromophore into the protein molecule. Reduction with dithiothreitol led to removal of the chromophore, indicating that incorporation was through the mixed disulfide.

Although there are no cysteine residues in native ribonuclease, it is known that thiols can be formed in certain protein degradative processes (Whitaker & Feeney, 1983). It was expected that in order for ribonuclease to undergo inactivation by disulfide exchange, thiols must be present in the mixture. On the other hand, no thiols should be present under conditions where the process is inhibited. To test this, ribonuclease was thermoinactivated at both pH 6 and 8 and in the presence and absence of 10 μM $CuCl_2$. Samples were removed periodically and assayed for free SH groups with Ellman's reagent. The results of the experiments conducted at pH 8 are shown in Figure 3A. It can be seen that thiols required for catalysis of the disulfide exchange reaction are indeed generated during thermoinactivation and that no thiols were present in the solution containing $CuCl_2$. This finding is consistent with the assumption that copper ion stabilizes the enzyme by catalyzing the removal of thiols from the thermoinactivation mixture. Qualitatively similar results were obtained when samples of ribonuclease were heated at pH 6. Ultrafiltration and sulfhydryl determination of ribonuclease that had been thermoinactivated at pH 8 revealed that around two-thirds of the thiols formed were in the low molecular weight fraction and that the remaining one-third were bound to the protein. It has been reported that both protein-bound and low molecular weight thiols, including HS^-, are generated as a result of alkaline decomposition of cystine residues via the β-elimination mechanism (Florence, 1980; Whitaker & Feeney, 1983). It will be shown in the next section that at neutral pH and 90 °C, destruction of ribonuclease's cystines also proceeds through this pathway. It is reasonable to assume, therefore, that this process generates the thiols that catalyze disulfide interchange—the major process contributing toward irreversible thermoinactivation of ribonuclease at neutral pH.

(*C*) *Heat-Induced Alterations in the Primary Structure of Ribonuclease.* It was shown in the previous section that at 90 °C and pH 6 the rate constant of thermoinactivation of ribonuclease after unscrambling of incorrectly paired disulfide bonds is 0.25 h^{-1}. This represents 45% of the overall rate constant. Similarly, at pH 8 and 90 °C, the rate constant of thermoinactivation after unscrambling is 4.0 h^{-1}, i.e., 17% of the overall value. Since the processes contributing to these remaining portions are not reversed by complete reduction and unfolding, they must involve changes in the primary structure.

In an effort to identify those processes, the effect of thermoinactivation on the amino acid composition of the enzyme was determined. Samples of native ribonuclease and of ribonuclease that had been thermoinactivated at either pH 6 or pH 8 for different periods of time were reduced and carboxymethylated and then subjected to acid hydrolysis and HPLC amino acid analysis. At pH 6, the only significant change in the amino acid composition during thermoinactivation is a decrease in *S*-(carboxymethyl)cysteine, which indicates that thermoinactivation at pH 6 is accompanied by destruction of cystine residues. Thermoinactivation at pH 8, on the other hand, is accompanied by two major changes in amino acid composition: destruction of cystines and a slower disappearance of lysine residues. Interestingly, more than one cystine residue is eventually destroyed, indicating that this process is not limited to a single, particularly labile site in the protein molecule.

To assess the magnitudes of the contribution of cystine destruction toward thermoinactivation of ribonuclease at pH 6 and 8, the time courses of thermoinactivation (after unscrambling of incorrect S–S bonds) at each pH were compared to those of destruction of cystine residues (Figures 4 and 5,

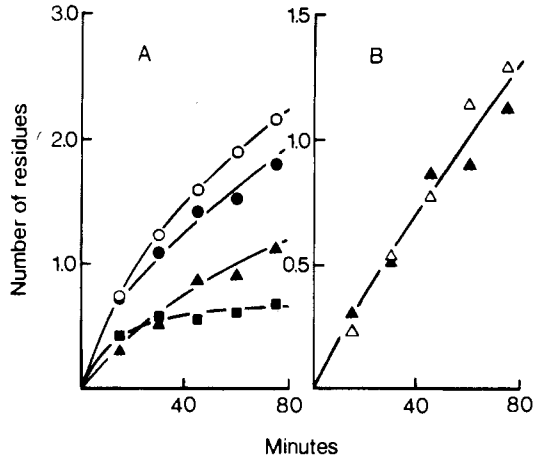


FIGURE 6: Fate of cystine and lysine residues destroyed during thermoinactivation of ribonuclease at pH 8 and 90 °C. (A) Time course of disappearance of cystine residues (○), of formation of dehydroalanines (■) and lysinoalanines (▲), and of the sum of dehydroalanine and lysinoalanine residues formed (●). (B) Time course of disappearance of lysine residues (△) and of formation of lysinoalanine residues (▲). Thermoinactivation conditions were the same as in Figure 2. Cystine, lysine, dehydroalanine, and lysinoalanine residues were determined as described in the text.

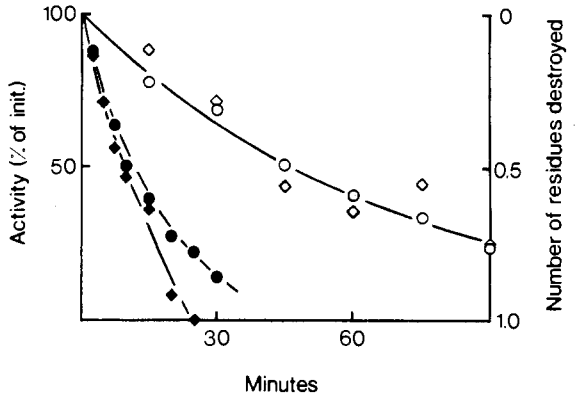


FIGURE 7: Stabilization of potential ribonuclease activity against thermoinactivation at pH 8 and 90 °C by reduction of cystine residues to cysteines. The time courses of loss of potential activity of reduced ribonuclease (○) and of the unmodified enzyme (●) were determined and compared to the time courses of destruction of cysteine residues (◇) and of cystine residues (◆), respectively. The potential activity of the reduced samples was determined after reoxidation under native conditions. Unmodified samples were assayed after unscrambling of S–S bonds by reduction/reoxidation as described under Experimental Procedures. Thermoinactivation of the reduced enzyme was carried out in the presence of 10 mM dithiothreitol. Other conditions were the same as in Figure 2.

respectively). On the basis of the highly plausible assumption that the destruction of any one cystine residue is sufficient to inactivate the enzyme, the rate constants of thermoinactivation due to destruction of cystine at each value of the pH can be estimated. At pH 6, the value obtained is 0.054 h^{-1}, which is approximately 10% of that for the overall thermoinactivation process. At pH 8, the rate constant of thermoinactivation due to cystine destruction is 2.8 h^{-1}, i.e., 12% of the overall value. Therefore, the destruction of cystine residues may play a significant role in thermoinactivation of ribonuclease at neutral pH. Because at pH 8 cystine destruction occurs at a greater rate relative to that of thermoinactivation due to changes in primary structure, this process was studied in detail at that pH value. Additional experiments were directed toward elucidation of the chemical mechanism of the observed destruction of cystines and toward confirmation of the assumption that cystine destruction actually causes inactivation of the enzyme.

The finding that destruction of cystine residues at pH 8 is accompanied by disappearance of lysines suggests that cystines undergo destruction via the β-elimination reaction (Nashef et al., 1977) and that the disappearance of lysine is due to the subsequent formation of lysinoalanine (Bohak, 1964). This process is the accepted pathway of alkaline destruction of cystine residues in proteins (Nashef et al., 1977; Whitaker & Feeney, 1983). The base-catalyzed abstraction of a proton β with respect to the disulfide bond results in cleavage of the cystine cross-link and formation of residues of dehydroalanine and thiocysteine. Dehydroalanine then can undergo an additional reaction with a lysine ε-amino group, yielding a new intramolecular cross-link, lysinoalanine (Bohak, 1964). It has been shown that hydrosulfide ion is among the products of breakdown of the thiocysteine residue (Gawron & Odstrchel, 1967; Florence, 1980).

To confirm that β-elimination is the mechanism responsible for destruction of cystine residues in ribonuclease at pH 8 and 90 °C, the kinetics of formation of dehydroalanine and lysinoalanine, two known products of β-elimination, were measured and compared to those of disappearance of cystine and lysine. Figure 6A depicts the time course of disappearance of cystine residues [determined as *S*-(carboxymethyl)cysteine using amino acid analysis] along with the simultaneous appearance of dehydroalanine and of lysinoalanine. Also shown is the sum of the number of lysinoalanines and dehydroalanines which, according to the reaction mechanism, should be equal to the number of cystines destroyed. It can be seen from the figure that this is very close to what was actually observed and hence that the major pathway of destruction of cystine residues at pH 8 and 90 °C is indeed the β-elimination reaction. In Figure 6B, the decrease in the number of lysine residues is compared with the increase in lysinoalanines. It can be seen that the two curves are similar, indicating that the cause of disappearance of lysine residues is their conversion to lysinoalanine.

To determine whether the observed destruction of cystines actually causes thermoinactivation of ribonuclease, the following approach was employed: Cystine residues of the enzyme were reversibly modified in order to alter their reactivity toward the β-elimination reaction. The effects of modification on loss of activity at 90 °C and pH 8 (determined after reversal of the modification) and on the destruction of potential cystines occurring under these conditions were then compared. Parallel changes in the reactivity of cystines and thermostability of the enzyme would provide a compelling indication that thermoinactivation is due to the destruction of cystines.

The reversible modifications employed were (i) reduction of cystine residues to cysteines (Figure 7) and (ii) cleavage of S–S bonds with trisodium phosphorothioate (Neumann et al., 1964). In both cases, modification resulted in substantial (5- and 2-fold, respectively) stabilization of both cystine residues and enzymatic activity. These findings indicate that destruction of cystines does not just coincide with loss of activity but is a cause of it.

The aforedescribed data not only show that β-elimination of cystine residues significantly contributes to irreversible thermoinactivation of ribonuclease at neutral pH but also reveal the origin of the thiols that catalyze disulfide scrambling of the enzyme.

(*D*) *Heat-Induced Alterations in the Primary Structure of Ribonuclease Not Detectable by Amino Acid Analysis.* There are several alterations in the primary structure that may contribute to irreversible thermoinactivation of enzymes but that cannot be detected by the HPLC amino acid analysis employed by us. These include (i) hydrolysis of peptide bonds,

Table I: Thermoinactivation of Ribonuclease due to Deamidation at pH 6 and 90 °C[a]

no. of amide residues hydrolyzed	sp act. (% of native)[a]	% distribution of deamidated species as function of thermoinactn time (h)[b]					
		0	2	4	6	8	10
0	100	95	27	6			
1	65	5	49	40	28	18	
2	38		18	39	39	32	29
3	19		7	14	28	34	55
4				2	5	16	16
	% inactn due to deamination[a]	0	32	50	64	69	79
	equiv of NH_3[d]	0.0	1.0	1.6	2.1	2.4	2.8

[a] The specific activity of each deamidated form of ribonuclease was determined by assay of samples isolated using preparative isoelectric focusing. [b] The percent distribution of deamidated species in each thermoinactivated sample was measured by using analytical isoelectric focusing and gel scanning densitometry. [c] The percent inactivation of each sample due to deamidation was then calculated by using this distribution and the specific activity of each form. [d] NH_3 released was calculated by assuming 1 equiv released per amide side chain hydrolyzed. All methods are as described in the text. Thermoinactivation conditions were the same as in Figure 1.

(ii) deamidation of Asn and/or Gln residues, (iii) racemization of amino acid residues, and (iv) destruction of Trp and Pro residues. Destruction of Trp can be ruled out since ribonuclease does not contain this amino acid. Racemization of amino acids has been ruled out on the basis of the literature data (Steinberg et al., 1984; Bada, 1985). Conventional amino acid analysis of samples of irreversibly thermoinactivated ribonuclease revealed that proline destruction had not taken place.

The possibility that peptide bond hydrolysis contributes to ribonuclease thermoinactivation at neutral pH was ruled out on the basis of SDS–polyacrylamide gel electrophoresis of native and thermoinactivated enzyme samples carried out under reducing conditions (Laemmli, 1970). No noticeable quantities of low molecular weight protein products were found in samples thermoinactivated at either pH 6 or pH 8. This finding was confirmed by using acid hydrolysis and amino acid analysis of low molecular weight fractions obtained by ultrafiltration through an Amicon YM2 membrane (1000 molecular weight cutoff).

The kinetics of deamidation of Asn and/or Gln residues of ribonuclease at neutral pH and 90 °C were determined by measuring the time course of the appearance of ammonia in solutions of the enzyme undergoing irreversible thermoinactivation at both pH 6 and pH 8. The results of these experiments are shown in panels A and B, respectively, of Figure 8. It can be seen that deamidation occurs at a substantial rate at both pH values.

In order to examine the effect of deamidation of ribonuclease on its catalytic activity, it was necessary to isolate the deamidated forms of the enzyme. The method employed was based on the decrease in the isoelectric point (p*I*) of the enzyme that results from conversion of amide side chains to carboxylate groups. Enzyme molecules that have undergone deamidation to different extents can therefore be separated on the basis of p*I* using isoelectric focusing. Determination of the contribution of deamidation to thermoinactivation of ribonuclease consisted of two steps. First, the time course of appearance of different deamidated forms of the enzyme (i.e., monodeamidated, dideamidated, etc.) was measured by using analytical isoelectric focusing. Then, the specific activity of each deamidated form was determined by using samples that had been isolated from isoelectric focusing gels run on a preparative scale. From these data, the kinetics of loss of activity due to deamidation can be calculated.

Time points from the thermoinactivation of ribonuclease at pH 6 were run on a nonequilibrium isoelectric focusing gel (O'Farrell et al., 1977), which then was stained with Coomassie Blue (Figure 9). It can be seen that as a result of

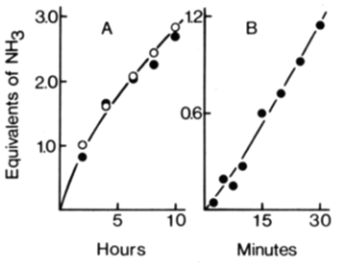


FIGURE 8: (A) Time course of deamidation of ribonuclease at pH 6 and 90 °C. (●) Number of equivalents of ammonia released, as determined directly by enzymatic assay; (○) calculated time course of ammonia release, based on the distributions of deamidated forms determined by isoelectric focusing and the assumption that 1 equiv of NH_3 is released per equivalent of monodeamidated ribonuclease, two per dideamidated, etc. Samples were heated in sealed ampules under conditions described in Figure 1. (B) Time course of deamidation of ribonuclease at pH 8 and 90 °C, as determined by enzymatic assay of ammonia released. Samples were heated in sealed ampules under conditions described in Figure 2.

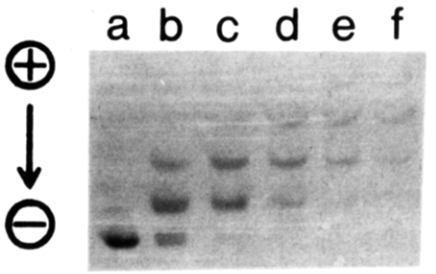


FIGURE 9: Deamidation of ribonuclease at pH 6 and 90 °C. Solutions of ribonuclease were heated for (a) 0, (b) 2, (c) 4, (d) 6, (e) 8, and (f) 10 h and then subjected to isoelectric focusing (O'Farrell et al., 1977) as described under Experimental Procedures. Thermoinactivation conditions were the same as in Figure 1.

heating, the native band gradually disappears and is replaced by a series of bands with decreasing p*I*'s. It is reasonable to assume that each of these new bands consists of protein that has undergone deamidation to a different extent; i.e., the second from the bottom contains monodeamidated ribonuclease, the third contains dideamidated, etc. The distribution of deamidated species in each sample was measured by using gel scanning densitometry. The assumption that the appearance of new bands is the result of deamidation was confirmed by comparison of a "predicted" time course of ammonia release, calculated by using the densitometry data, with the actual measured time course (Figure 8A).

Preparative isoelectric focusing of thermoinactivated ribonuclease revealed that deamidation at pH 6 does in fact result in substantial loss of activity (Table I). A time course of

Table II: Mechanism of Irreversible Thermoinactivation of Ribonuclease at 90 °C as a Function of pH[a]

process	rate constant (h^{-1}) at pH 4	pH 6	pH 8
overall obsd thermoinactn	0.13	0.56	23.4
thermoinactn due to individual mechanisms			
peptide bond hydrolysis	0.10	0	0
deamidation of Asn and/or Gln residues	0.024	0.15	0.8
thiol-catalyzed disulfide interchange	0	0.31	19.4
β-elimination of cystine residues	0	0.054	2.8

[a] At each pH, the rate constants of thermoinactivation due to each individual process are given along with that of the overall observed loss of activity.

thermoinactivation at pH 6 due to deamidation can be calculated (Table I) by using these data and the distributions of the deamidated species in each time point. The rate constant of thermoinactivation due to deamidation was determined to be 0.15 h^{-1}, which is 27% of the rate constant of the overall thermoinactivation process. Therefore, deamidation of Asn and/or Gln residues of ribonuclease is the major thermoinactivation process taking place at pH 6 and 90 °C that involves alteration of the primary structure of the enzyme and, together with destruction of cystine residues, can account for essentially all of the lost enzyme activity that is not regained by unscrambling of incorrect disulfide pairings.

To estimate the contribution of deamidation toward thermoinactivation of ribonuclease at pH 8, the percent of the initial activity lost per amide group hydrolyzed at pH 6 was determined (assuming an additive relationship). This value is 27% inactivation per equivalent of NH_3 released. By use of this value, and the time course of ammonia evolution at pH 8 and 90 °C, the estimated rate constant of thermoinactivation of ribonuclease due to deamidation under these conditions was 0.8 h^{-1} (i.e., 3% of the overall rate constant).

(*E*) *Summary.* The findings presented here provide a complete description of the mechanism of irreversible thermoinactivation of ribonuclease at neutral pH. The major process, thiol-catalyzed intramolecular interchange of disulfide bonds, constitutes 55% and 83% of the overall observed thermoinactivation at pH 6 and 8, respectively. Two other mechanisms, deamidation of Asn and/or Gln residues and β-elimination of cystines, are responsible for 27% and 10% of the overall process at pH 6 and for 3% and 13% at pH 8, respectively. These results are shown in Table II and afford an answer to the following question: "Why does ribonuclease undergo irreversible thermoinactivation at 90 °C and neutral pH?"

Irreversible Thermal Inactivation of Ribonuclease at Acidic pH. Experiments aimed at elucidation of the mechanism of irreversible thermoinactivation of ribonuclease under mildly acidic conditions were mainly carried out at pH 4 and 90 °C. Thermoinactivation of ribonuclease under these conditions could not be reversed by reduction/reoxidation and therefore must be due to processes that involve changes in the primary structure. Disulfide bond interchange, which is the major thermoinactivation process at neutral pH, does not contribute toward thermoinactivation at pH 4 to any significant extent.

To identify the changes in the primary structure of ribonuclease that cause its thermoinactivation at mildly acidic pH, the effect of heating on the amino acid composition of the enzyme was investigated. HPLC amino acid analysis (Fernstrom & Fernstrom, 1981) of acid hydrolysates of reduced and carboxymethylated (Bibring & Baxandall, 1978) samples of native and thermoinactivated ribonuclease revealed that no significant changes accompany irreversible thermo-

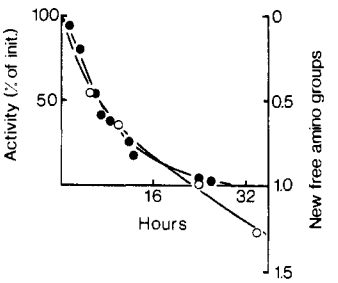


FIGURE 10: Time course of irreversible thermoinactivation of ribonuclease and of hydrolysis of peptide bonds at pH 4 and 90 °C. (●) Percent of initial enzyme activity; (○) number of peptide bonds hydrolyzed per molecule as measured by determination of new free amino groups using trinitrobenzenesulfonate titration of reduced and carboxymethylated samples. Thermoinactivation was carried out at a protein concentration of 0.67 mg/mL in 0.01 M formate buffer containing 0.15 M KCl.

inactivation at pH 4 and 90 °C. There is no appreciable decrease in *S*-(carboxymethyl)cysteine, indicating that destruction of cystine residues, which is responsible in part for ribonuclease thermoinactivation at neutral pH, does not occur under mildly acidic conditions.

We then examined possible changes in primary structure that may contribute to thermoinactivation of enzymes but that cannot be detected by acid hydrolysis followed by HPLC analysis. Racemization of amino acid residues and destruction of Trp residues have again been ruled out. Determination of proline by conventional amino acid analysis (Hare, 1975) revealed that Pro residues were not destroyed during thermoinactivation of ribonuclease at 90 °C and pH 4. This leaves two processes that could not be ruled out: hydrolysis of peptide bonds and deamidation of Asn and/or Gln residues.

The possibility that peptide bond cleavage contributes to ribonuclease thermoinactivation at pH 4 was initially evaluated by using SDS–polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970). Staining with Coomassie Blue revealed that thermoinactivation did result in a significant decrease in the intensity of the band in the native position and in the formation of low molecular weight products.

A quantitative approach to evaluation of the role of peptide bond hydrolysis in ribonuclease thermoinactivation at pH 4 is the spectrophotometric determination of new free amino groups (Fields, 1971) that result from the formation of new N-terminal amino acid residues at the sites of cleavage. Enzyme samples were reduced and carboxymethylated prior to titration in order to expose all 11 free amino groups, some of which are not accessible to the reagent in native ribonuclease (Fields, 1971). The time course of appearance of new free NH_2 groups was thus determined in reduced and carboxymethylated enzyme samples that had been removed at various stages of thermoinactivation at pH 4. Figure 10 shows the kinetics of the appearance of new free amino groups and of the loss of catalytic activity. It can be seen that hydrolysis of approximately one peptide bond coincides with inactivation of the enzyme. This finding suggests that irreversible thermoinactivation of ribonuclease at pH 4 may be due to cleavage of peptide bonds. The observation that the appearance of new free amino groups continues beyond 1 mol equiv indicates that hydrolysis is not restricted to a single site in the protein molecule.

The accuracy and precision of kinetic analyses of peptide bond hydrolysis in ribonuclease using titration of free amino groups are subject to the following limitation: since native ribonuclease already contains 11 free amino groups, the hydrolysis of 1 peptide bond results in an increase in free amino

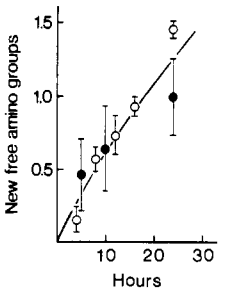


FIGURE 11: Time course of appearance of new free amino groups in acetylated and unmodified ribonuclease at pH 4 and 90 °C. Samples of ribonuclease (●) and acetylated ribonuclease (○) were heated, reduced and carboxymethylated, and then assayed for free amino groups with trinitrobenzenesulfonate as described under Experimental Procedures. Error bars indicate the standard deviation of the amino group determinations, performed in triplicate. Thermoinactivation conditions were the same as in Figure 10.

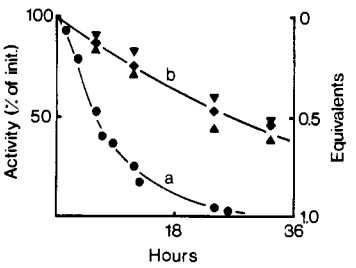


FIGURE 12: Time course of irreversible thermoinactivation of ribonuclease and of cleavage of the Asp_{121}–Ala_{122} peptide bond at pH 4 and 90 °C. (a) Percent of initial enzyme activity (●); (b) number of equivalents of C-terminal tripeptide amino acids in the hydrolysates of YM2 ultrafiltrates: Ala (▼); Ser (♦); Val (▲). Experimental conditions were the same as in Figure 10.

groups of less than 10%. Therefore, it was desirable to lower the initial titer of amino groups in ribonuclease and thereby increase the percent change in the number of free amino groups that results from hydrolysis of peptide bonds. To this end, ribonuclease was modified with acetic anhydride. Acetylation of ribonuclease in the presence of pyrophosphate, a competitive inhibitor (Giese & Vallee, 1972), resulted in the blockage of between eight and nine free amino groups, with only a minor (about one-third) loss of activity and no change in the time course of thermoinactivation at pH 4. With this modified enzyme, hydrolysis of a single peptide bond will now cause an increase in free amino groups of 35–50%. The time course of appearance of new free amino groups in the acetylated ribonuclease as a result of heating at pH 4 and 90 °C was then determined and compared with that obtained with the unmodified enzyme (Figure 11). The error bars indicate the standard deviation of trials performed in triplicate. It can be seen that the two curves are in reasonable agreement and that acetylation does result in a heightened precision. The possibility that new free amino groups might arise in the acetylated enzyme as a result of deacetylation was ruled out by assaying the enzyme solution for acetate (Beutler, 1984) before and after heating. Therefore, it is concluded that measurement of the time course of peptide bond hydrolysis using titration of new free amino groups is indeed valid, in spite of the large number of free amino groups present in the unheated enzyme.

In the above experiments, samples were passed through a Sephadex G-10 column following reduction and carboxymethylation. Hence, any small fragments (M_r <700) released from ribonuclease (resulting from cleavage of peptide bonds near the termini of the molecule) during thermoinactivation could not be detected by this method. An approach was devised to determine whether such small fragments were in fact cleaved from the protein molecule.

Samples of ribonuclease at various stages of thermoinactivation were placed in an Amicon ultrafiltration cell with a YM2 (1000 molecular weight cutoff) membrane. The fraction that passed through the membrane was acid hydrolyzed and analyzed by HPLC. It was found that three amino acids appeared in the hydrolysate of the low molecular weight fraction in approximately equimolar quantities. These were alanine, serine, and valine—the three C-terminal amino acids of ribonuclease (Richards & Wyckoff, 1971). These results indicate that a cleavage site not detectable by the trinitrobenzenesulfonate method is the peptide bond linking Asp_{121} and Ala_{122}. A first-order rate constant of hydrolysis at this site was determined to be 0.027 h^{-1}, approximately 20% of the rate constant of irreversible thermoinactivation of ribonuclease at pH 4. The time course of the appearance of these three amino acids in the hydrolysate of the low molecular weight fraction and of ribonuclease thermoinactivation is shown in Figure 12.

Heating of proteins in acidic solutions results in preferential cleavage of peptide bonds involving the carboxyl group of Asp residues (Inglis, 1983). Given that release of the C-terminal tripeptide of ribonuclease involves scission of this type of bond (Asp_{121}–Ala_{122}), it seemed likely that the hydrolysis of peptide bonds measured with trinitrobenzenesulfonate also occurs at sites involving Asp residues, of which ribonuclease has a total of five. This hypothesis was evaluated by subjecting the thermoinactivated enzyme to end group analyses.

Hydrazinolysis (Braun & Schroeder, 1967) of S-carboxymethylated samples of native and thermoinactivated ribonuclease revealed the appearance of significant quantities of only one new C-terminal residue—aspartic acid (Figure 13A). Digestion of thermoinactivated samples with carboxypeptidase Y (Hayashi, 1977) also resulted in preferential release of aspartic acid (Figure 13B). The five amino acids released in the next largest amounts—threonine, methionine, leucine, alanine, and phenylalanine—are all located either one or two residues before Asp residues in ribonuclease. On the basis of the specificity of carboxypeptidase Y, it is expected that these amino acids would be quickly released following enzymatic cleavage of the new C-terminal Asp residues. Dansyl chloride end group analysis (Fleischman, 1973) of thermoinactivated samples revealed the presence of new N-terminal Ser and Val residues. Both of these amino acids are located adjacent to aspartic acid residues (Asp_{14}-Ser_{15} and Asp_{53}-Val_{54}) and hence would be expected to appear as new N-termini if cleavage occurs at the carboxyl group of Asp residues. These results indicate that irreversible thermoinactivation of ribonuclease at pH 4 and 90 °C is accompanied by cleavage of peptide bonds on the carboxyl side of several different Asp residues.

The rate constant of irreversible thermoinactivation of ribonuclease due to peptide bond hydrolysis can be estimated on the basis of the plausible assumption that hydrolysis of any one of the labile peptide bonds (those adjacent to Asp residues) results in loss of catalytic activity. Analysis of the time course of appearance of new free amino groups in acetylated ribonuclease at pH 4 and 90 °C yielded a value of 0.075 h^{-1} for the rate constant of thermoinactivation due to hydrolysis of peptide bonds not located near the ends of the protein molecule. This analysis required two additional assumptions: (i) that the peptide bond hydrolysis detected by titration of free amino groups can take place at any one of four sites [the basis of this assumption is that ribonuclease has four Asp residues aside from the one near the C-terminus (Asp_{12})] and (ii) that all four sites are comparably susceptible to hydrolysis.

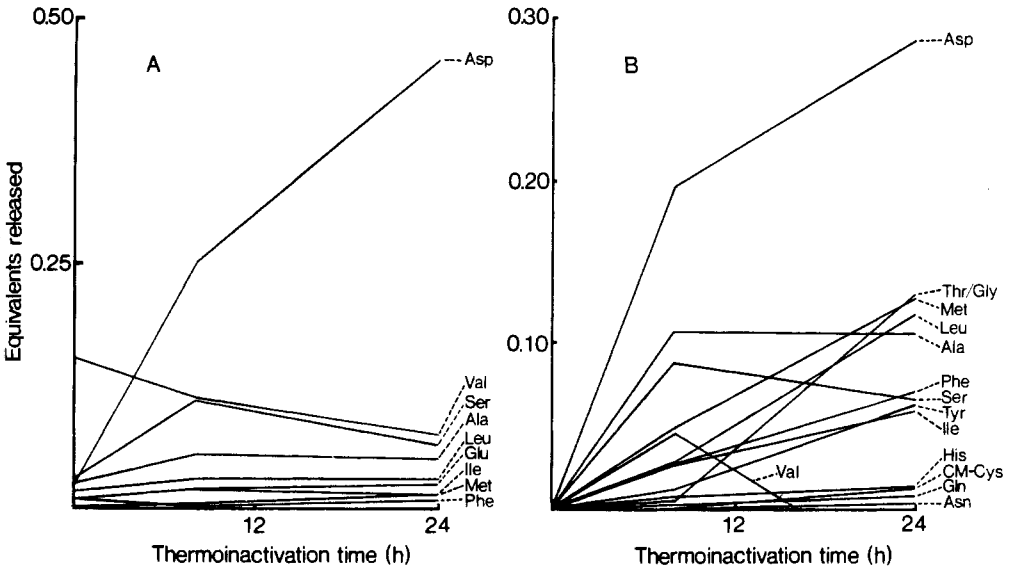


FIGURE 13: Effect of irreversible thermoinactivation of ribonuclease at 90 °C and pH 4 on the products of hydrazinolysis of the enzyme and digestion of the enzyme with carboxypeptidase Y. (A) New C-terminal amino acids were identified by hydrazinolysis of S-carboxymethylated samples of ribonuclease that had been inactivated for 0, 8, and 24 h (100, 28, and 2.5% of residual activity, respectively). Products were determined by HPLC. Thermoinactivation conditions were identical with those in Figure 10. See the text for the hydrazinolysis procedure. (B) S-Carboxymethylated samples of ribonuclease that had been thermoinactivated for 0, 8, and 24 h (100, 28, and 2.5% of residual activity, respectively) were digested with carboxypeptidase Y and the amino acids released determined by HPLC. Results from the unheated sample (t = 0) were subtracted from those of thermoinactivated samples to correct for the presence of the native C-terminal sequence. Thermoinactivation conditions were the same as in Figure 10.

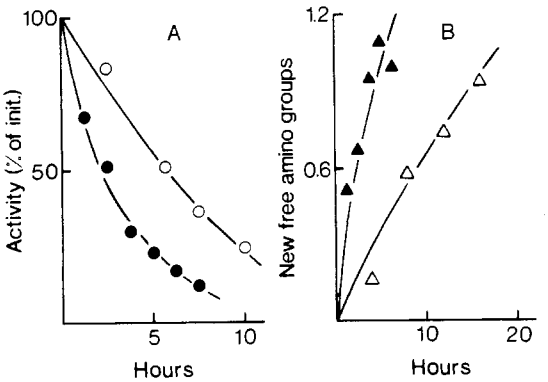


FIGURE 14: Peptide bond hydrolysis and irreversible thermoinactivation of acetylated ribonuclease at pH 3 and 4 and 90 °C. (A) Time course of irreversible thermoinactivation at pH 3 (●) and at pH 4 (○). (B) Time course of appearance of new free amino groups at pH 3 (▲) and at pH 4 (△). Free amino groups were determined by spectrophotometric titration of reduced and carboxymethylated samples with trinitrobenzenesulfonate, as described under Experimental Procedures. Thermoinactivation at pH 3 was carried out in 0.01 M citrate buffer containing 0.15 M KCl at a protein concentration of 40 μM. Thermoinactivation at pH 4 was carried out as described in Figure 10.

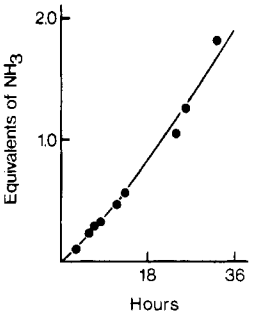


FIGURE 15: Time course of deamidation of ribonuclease at pH 4 and 90 °C as measured by enzymatic determination of ammonia released. Samples were heated in sealed ampules under conditions described in Figure 10.

At pH 4 and 90 °C, the rate constant of hydrolysis of the C-terminal tripeptide of ribonuclease was found to be 0.027 h^{-1}. If this value is combined with that determined above for thermoinactivation due to hydrolysis at the other four sites, a rate constant of thermoinactivation of ribonuclease due to peptide bond hydrolysis of 0.10 h^{-1} is obtained. This value is 77% of the overall rate constant of thermoinactivation observed under these conditions. Therefore, the hydrolysis of peptide bonds adjacent to Asp residues is the major process contributing toward ribonuclease thermoinactivation at pH 4.

The above conclusion is, of course, based on the assumption that the hydrolysis observed actually causes inactivation of the enzyme. To strengthen this assumption experimentally, a modification of the system was sought that would significantly change the rate of peptide bond hydrolysis. If a similar change in the kinetics of thermoinactivation was also observed, this would provide an indication that the two processes were linked. The modification employed was to change the pH of the system from 4 to 3. The time courses of thermoinactivation of acetylated ribonuclease and of the appearance of new free amino groups at pH 3 and 90 °C were determined and compared with those of the same processes at pH 4 (Figure 14). This change in pH resulted in a greater than 2-fold increase in the rate constant of thermoinactivation and a similar increase in the rate of appearance of new free amino groups. The similarlity of the pH dependences of thermoinactivation and peptide bond hydrolysis provides a strong indication that the latter process results in inactivation of the enzyme.

Deamidation of Asn and/or Gln residues of ribonuclease occurs at a significant rate at pH 4 and 90 °C (Figure 15). On the basis of the effects of deamidation at pH 6 on ribonuclease activity, a rate constant of thermoinactivation due to deamidation at pH 4 and 90 °C has been estimated. From the time course of NH_3 evolution at pH 4 (Figure 15) and the approximation that 27% of the initial activity is lost with each equivalent of ammonia released, a value of 0.024 h^{-1} has been obtained. This is 19% of the overall observed rate constant of thermoinactivation. Therefore, deamidation is responsible for a considerable portion of the thermoinactivation of ribo-

nuclease under mildly acidic conditions and, together with peptide bond hydrolysis, can essentially account for the entire overall observed irreversible thermoinactivation at pH 4.

The above findings provide a complete quantitative description of the mechanism of irreversible thermoinactivation of ribonuclease at pH 4. The major process, hydrolysis of peptide bonds at Asp residues, constitutes 77% of the overall observed thermoinactivation under these conditions. A second process, deamidation of Asn and/or Gln residues, is responsible for 19% of the overall process. The corresponding rate constants are presented in Table II, and the two processes combined afford an answer to the following question: "Why does ribonuclease undergo irreversible thermoinactivation at 90 °C and pH 4?"

Conclusions

We have revealed that irreversible thermoinactivation of ribonuclease at pH 6 and 8 is caused by a combination of disulfide interchange, β-elimination of cystine residues, and deamidation of Asn and/or Gln residues; at pH 4, the loss of activity is caused by a combination of hydrolysis of peptide bonds at Asp residues and deamidation of Asn and/or Gln residues. These four inactivating processes (Table II) are virtually the same as those previously found to bring about irreversible thermoinactivation of hen egg white lysozyme (Ahern & Klibanov, 1985). The three heat-induced reactions leading to changes in the primary structure (deamidation, hydrolysis, and β-elimination) are identical in the two enzymes. The fourth process, called "formation of incorrect (scrambled) structures" for lysozyme, was mechanistically investigated in detail in this work and found in the case of ribonuclease to be thiol-catalyzed interchange of S–S bonds. The commonality of the uncovered thermoinactivating processes for two unrelated enzymes strongly suggests that the same reactions will also take place in, and likely inactivate, other enzymes at high temperatures.

It should be stressed that in no case did we detect the presence of a single, particularly labile amino acid residue whose destruction would lead to enzyme inactivation. To the contrary, in each inactivating process (Table II) several amino acid residues were involved. For example, in the case of heat-induced hydrolysis of peptide bonds at Asp residues at pH 4, cleavage occurs at comparable rates at at least three different loci. The time course of β-elimination of disulfide bonds at pH 8 represents a smooth curve well beyond destruction of two out of four total cystine residues present and shows no signs of slowing down after one residue is destroyed (Figure 6). Analogous data were obtained for lysozyme (Ahern & Klibanov, 1985). Similarly, the time courses of heat-induced ammonia evolution at pH 4 both for lysozyme (Ahern & Klibanov, 1985) and for ribonuclease smoothly proceed beyond at least 10 Asn and/or Gln residues, thus ruling out the existence of 1 or 2 that are particularly susceptible to deamidation. All these results indicate that the sensitivity of a given amino acid residue to thermal destruction is not dramatically affected by its surroundings. The apparent lack of a significant effect of the protein secondary and tertiary structures was expected since at temperatures as high as 90–100 °C most of these structures are destroyed (Tanford, 1968; Lapanje, 1978). More surprisingly, the primary structure (i.e., the immediate neighbors in the polypeptide chain) does not have a dramatic influence either, at least in the direction of labilization. Therefore, since the uncovered thermoinactivating processes (Table II) are basic chemical reactions, they point to the upper limit of thermal stability of proteins.

Knowledge of what causes irreversible thermoinactivation of enzymes is conducive to rational approaches to enzyme stabilization. For instance, in the case of ribonuclease, such a simple measure as an addition of 10 μM Cu^{2+} resulted in a significant enhancement of thermal stability of the enzyme due to elimination of the thiols catalyzing disulfide interchange—the major cause of ribonuclease inactivation at 90 °C and neutral pH. Our data are also instructive for the development of general strategies of enzyme stabilization by protein engineering. First, it appears that one such strategy recently proposed, incorporation of additional S–S bridges (Perry & Wetzel, 1984), is not generally applicable, at least at neutral and alkaline pH, because it will make genetically redesigned enzymes more prone to such damaging processes as β-elimination of cystine residues and scrambling due to disulfide exchange. Second, prudent strategies should include replacement of "weak links" in protein molecules, e.g., Asp residues or Asn/Gln residues with other amino acid residues that will not undergo the corresponding destruction reactions.

It is tempting to inquire whether nature in fact has followed some of the aforementioned strategies. Comparison of amino acid compositions of mesophilic vs. thermophilic microorganisms (Argos et al., 1979) reveals no such evidence. However, an important clarification is in order here. There are two fundamentally different types of enzyme thermostability. The first one deals with the ability of an enzyme molecule to retain its catalytically active conformation (i.e., N as opposed to U in eq 1). Nature is keenly concerned about this type of stability in thermophiles where it is required to have catalytically active enzymes at high temperatures. Nature's strategy to secure such stability is to rigidify the enzyme molecule by introducing additional noncovalent interactions, e.g., electrostatic bonds (Perutz, 1978). Most studies on the biochemistry of thermophilic behavior (Zuber, 1976; Friedman, 1978; Argos et al., 1979) have been devoted to this type of thermostability. On the other hand, in this study we have addressed the second type of enzyme thermostability, namely, the ability to preserve the catalytic activity (or potential catalytic activity) over prolonged periods of time. It is not immediately clear from our data whether rigidification of enzyme molecules will be helpful in this respect; however, it is quite possible that in tightly folded thermophilic proteins at least some of the thermolabile residues (Table II), located in the interior of the macromolecule, will be inaccessible to water species (required for all four thermodeterioration reactions uncovered) and hence more thermoresistant. Nature probably does not have to deal with the issue of maintaining the enzymatic activity at high temperatures for a long time, as it seems easier to simply turn enzymes over faster in thermophiles vs. mesophiles using the accelerated proteolytic degradation/biosynthesis route. However, for the biochemical engineer, it is not feasible to replace the enzyme in the reactor every few hours, and hence both types of thermostability have to be dealt with. Since reversible protein unfolding occurs very quickly, if it has not taken place at once, then it will not happen later either. Therefore, the subsequent enzyme inactivation with time can be attributed exclusively to irreversible thermoinactivation—the subject of this investigation.

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Registry No. Ribonuclease, 9001-99-4.

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