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Regeneration of Ribonuclease A from the Reduced Protein. 1. Conformational Analysis of the Intermediates by Measurements of Enzymatic Activity, Optical Density, and Optical Rotation†

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ABSTRACT: Native bovine pancreatic ribonuclease A was regenerated from the fully reduced protein by 5×10^{-4} M reduced and oxidized glutathiones at pH 7.70–7.85 and 22–24 °C. The ensemble of all species except the fully reduced and the completely native proteins is designated as a set of "intermediates". The reshuffling of incorrectly paired disulfide bonds could be stopped at various times by lowering the pH to 4–5 to prepare samples of partially regenerated ribonuclease A which consisted of the intermediates *and* the completely regenerated native species. The partially regenerated material was identified by its enzymatic activity. The temperature dependence of the initial velocity of hydrolysis of the substrate C>p by these proteins, which is a probe of the thermodynamic properties of the active site, was measured at pH 5.00. The thermodynamic parameters T_m, $\Delta H°(T_m)$, and $\Delta S°(T_m)$ for thermal unfolding of fully and partially regenerated ribonuclease A (enzymatic activity in the range of 0.6–100%) were the same as those of the native protein. If the intermediates had been enzymatically active, they would have exhibited different thermodynamic properties than native ribonuclease A because of their wrongly paired disulfide bonds; since no such intermediates with different thermodynamic properties were detected, it is concluded that the intermediates are enzymatically inactive. The thermodynamic properties of the native and the fully or partially regenerated protein (enzymatic activity in the range of 2–100%) were also studied by measuring the temperature dependence of the difference optical density at 287 nm and of the difference optical rotation at 436 nm, at pH 4.00; these quantities reflect conformational changes of the environments of tyrosine residues and of the backbone, respectively. Fully or partially regenerated ribonuclease A (enzymatic activity in the range of 20–100%) exhibited the same thermodynamic parameters, T_m, $\Delta H°(T_m)$ and $\Delta S°(T_m)$, as observed for native ribonuclease A. The results demonstrate that the dominant conformations of the intermediates are disordered. Since, however, partially regenerated ribonuclease A (enzymatic activity in the range of 10–90%) starts to melt at a lower temperature than does native ribonuclease A, the presence of a small fraction of intermediates having an ordered structure [but with a lower transition temperature ($T_m \sim 40$ °C) than that ($T_m \sim 54$ °C) of native ribonuclease A at pH 4.0] is indicated.

Native bovine pancreatic ribonuclease A (RNase A)[1] has been regenerated from the fully reduced protein by air (Anfinsen et al., 1961; Epstein et al., 1962; Ahmed et al., 1975; Takahashi & Ooi, 1976; Takahashi et al., 1977; Chavez & Scheraga, 1977), by glutathione (Hantgan et al., 1974; Ahmed et al., 1975; Schaffer et al., 1975; Creighton, 1977, 1979; Chavez & Scheraga, 1980a), and in the presence of a microsomal enzyme (Goldberger et al., 1964). The present series of papers is devoted to the conformational analysis of the intermediates present during regeneration by glutathione.

Glutathione is a convenient reagent for studying the regeneration process because reoxidation of the sulfhydryl groups is almost complete immediately after addition of glutathione, and reshuffling of wrongly paired disulfide bonds among the reoxidized intermediates, and ultimately to fully regenerated

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[1] Abbreviations used: RNase A, bovine pancreatic ribonuclease A; DTT, DL-dithiothreitol; C>p, cytidine cyclic 2′,3′-phosphate; EDTA, ethylenediaminetetraacetic acid; OD_{275}, optical density at 275 nm; ΔOD_{287}, difference optical density at 287 nm; $v_i(T)$, initial velocity at temperature T; $\Delta[\alpha]_{436}$, difference optical rotation, i.e., optical rotation at 436 nm minus a constant value to bring it on scale; $(\Delta OD_{287})_{F-U}$, the difference of ΔOD_{287} between the thermally folded and unfolded states of RNase A at the transition temperature; $(\Delta[\alpha]_{436})_{F-U}$, the difference of $\Delta[\alpha]_{436}$ between the thermally folded and unfolded states of RNase A at the transition temperature.

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molecules, then proceeds slowly (Hantgan et al., 1974; Ahmed et al., 1975); i.e., the reoxidation and reshuffling processes can be studied separately. Moreover, the fact that only intramolecular disulfide bonds are formed (Creighton, 1977) and that the final product shows a high yield (~92%) of regeneration of enzymatic activity makes it feasible to analyze data on the reshuffling step.

Recently, Creighton (1979) carboxymethylated the intermediates and succeeded in fractionating them. In order to fractionate the intermediates that contain one, two, three, or four disulfide bonds and separate them from native RNase A, he found it necessary to modify the cysteine residues with the charged carboxymethyl group. Without carboxymethylation, the intermediates would have the same net charge, and it would not be possible to fractionate them by ion-exchange chromatography. While enabling the intermediates to be fractionated, carboxymethylation, however, would be expected to change their conformations (Gotto & Hamaguchi, 1979), as pointed out by Chavez & Scheraga (1980b), who found that the content of native conformation in reduced RNase A and in reduced carboxymethylated RNase A was quite different. Thus, the conformational analysis of the intermediates must be carried out with unmodified material, even though it is not fractionated.

It is necessary to examine the *temperature dependence* of the enzymatic activity in order to distinguish the active site of the intermediates from that of the native protein, since the two could have different thermodynamic properties because of their different disulfide pairings; thus, it is possible to assign the enzymatic activity of partially regenerated ribonuclease A either to the intermediates or to the *fully* regenerated protein; the results of these experiments are compared with those of Creighton (1979). Similarly, if one examines the *temperature dependence* of ΔOD_{287}, which reflects conformational changes of the environments of tyrosine residues, and of $\Delta[\alpha]_{436}$, which reflects primarily conformational changes of the backbone, it is possible to obtain information about the conformations of the intermediates.

Experimental Section

Materials. Bovine pancreatic ribonuclease A, obtained from Sigma Chemical Co., type II-A, was purified on a carboxymethylcellulose column (Taborsky, 1959). Tris base, reduced and oxidized glutathione, and DL-dithiothreitol (DTT) were purchased from Sigma Chemical Co. Ultrapure urea was obtained from Schwarz/Mann and was used without further purification. Barium cytidine cyclic 2′,3′-phosphate (C>p) was purchased from P-L Biochemicals, Inc. 5,5′-Dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) was purchased from Aldrich Chemical Co., Inc. Sephadex G-25 (with fine and superfine particle size) was obtained from Pharmacia Fine Chemicals. Carboxymethylcellulose (CM 52) was purchased from Whatman Ltd.

(*1*) *Reduced RNase A.* Since DTT is a strong reducing agent [with an equilibrium constant of 1.3×10^4 for the reduction of cystine (Cleland, 1964)], native RNase A (1.46 mM) was completely reduced by 65 mM DTT in 8 M urea and 0.1 M Tris-HCl buffer (pH 8.0) over a period of 2 h at room temperature (Iyer & Klee, 1973). Reduced RNase A was isolated on Sephadex G-25 (fine; 2.5 × 60 cm) equilibrated with 0.6% acetic acid at room temperature (White, 1967) and was lyophilized, the acetic acid serving to prevent oxidation of sulfhydryl groups (Anfinsen & Haber, 1961). The number of sulfhydryl groups in the reduced RNase A, as determined spectrophotometrically with Ellman's reagent (Ellman, 1959; Taniuchi, 1970), was found to be 8.0 ± 0.2.

(*2*) *Fully or Partially Regenerated RNase A.* The lyophilized reduced RNase A was dissolved in 0.6% acetic acid, which was 10^{-4} M in EDTA, and the pH of the solution was adjusted to 7.70–7.85 with 1.0 M Tris base. The rate of regeneration is insensitive to pH in this pH range (Hantgan et al., 1974) because of the presence of an excess of ionized reduced glutathione. The oxidation of reduced RNase A was started by adding an equimolar mixture of reduced and oxidized glutathione. The final concentrations of each reagent in the reaction solution were 8.4×10^{-5} M reduced RNase A, 5.0×10^{-4} M reduced and oxidized glutathione, 10^{-4} M EDTA, and 0.1 M Tris–acetate buffer (pH 7.70–7.85). After rapid reoxidation with a half-time of less than 1 min (Hantgan et al., 1974), the reshuffling process was stopped at various times by lowering the pH to 4–5 with glacial acetic acid. The partially regenerated RNase A was isolated from glutathione, EDTA, and Tris on Sephadex G-25 (superfine; 2.5 × 30 cm) equilibrated with 0.6% acetic acid at room temperature. The products were lyophilized and stored at −70 °C for less than 4 weeks. The enzymatic activity of the products did not change during this storage period. The partially regenerated RNase A was identified by its enzymatic activity against the substrate C>p.

In the preparation of *fully* regenerated RNase A, the treatment with glutathione was allowed to proceed for 24 h, producing a maximum enzymatic activity of 92%. This material was purified by chromatography on a carboxymethylcellulose column (Taborsky, 1959). The fractions of the fully regenerated RNase A from the carboxymethylcellulose column were desalted on Sephadex G-25, lyophilized, and stored at 4 °C for less than 4 weeks. The enzymatic activity of this *purified* fully regenerated RNase A was the same as that of native RNase A.

Protein Concentration. A Zeiss Model PMQ II or Cary Model 14 spectrophotometer was used to determine the concentration of RNase A. Absorption measurements and nitrogen analysis revealed that an isosbestic point exists at 275 nm with a value of $\epsilon_{275} = 9300$ M^{-1} cm^{-1} for native and *partially regenerated* RNase A in 0.6% acetic acid. In contrast to the results of others, in which 275 nm (with $\epsilon_{275} \sim 9300$ M^{-1} cm^{-1}) is also an isosbestic point for native and *reduced* RNase A (White et al., 1961; Hantgan et al., 1974; Schaffer, 1975), we found a lower value of $\epsilon_{275} = 8100 \pm 400$ M^{-1} cm^{-1} for reduced RNase A in 0.6% acetic acid, based on nitrogen analysis and absorption measurements. The lower value of ϵ_{275} for reduced RNase A compared to that for native RNase A seems to arise from the cleavage of disulfide bonds ($\epsilon_{275} \sim 140$ for L-cystine and $\epsilon_{275} \sim 0.3$ for L-cysteine) (Lamfrom & Nielsen, 1958) and from the change of the extinction coefficient of tyrosine residues close to disulfide bonds when the latter are reduced.

Measurement of Enzymatic Activity. The enzymatic activities of native and the fully or partially regenerated RNase A were measured spectrophotometrically by using C>p as a substrate (Crook et al., 1960).

The temperature dependence of the initial velocity of hydrolysis of C>p by native and partially or fully regenerated RNase A was measured, by using conditions similar to those of Matheson & Scheraga (1979), as follows. The protein was dissolved in degassed 0.6% acetic acid, and its concentration was measured by using $\epsilon_{275} = 9300$ M^{-1} cm^{-1}. The concentration of the protein was adjusted to give an initial velocity comparable to that of native RNase A at a concentration of either 1.4×10^{-7} or 3.3×10^{-7} M. The pH of the solution was adjusted roughly to 5.0 ($I = 0.064$ M) with solid Tris base,

and a fine adjustment was achieved with a negligible amount of 1 M Tris base to minimize the change in the volume of the solution; hence, the change in the concentration of the protein during the adjustment of pH was negligible. Then 3.0 mL of the protein solution was incubated in a stoppered UV cell for 14 min to obtain thermal equilibrium (Brandts & Hunt, 1967). The temperature was controlled by a Haake Type F circulating bath with an accuracy of ±0.1 °C, and a calibrated thermistor probe was used to measure the temperature in the cell. After the addition of 10 μL of C>p solution (60 mg/mL), the initial velocity was measured by following the change in absorbance at 286 nm on a Cary Model 14 spectrophotometer with a scale of 0.0 to 0.1. At this concentration of substrate, the enzyme is saturated (Matheson & Scheraga, 1979); thus, we measured only the maximum initial velocity and not the Michaelis constant.

In the following paper (Konishi & Scheraga, 1980), a study of the intermediates by nuclear magnetic resonance (NMR) spectroscopy is described. In order to prevent broadening of the C(2)- and C(4)-proton resonances of the His-48 residue in that study, an acetate buffer was used (Markley, 1975); i.e., 0.6% acetic acid was used as a solvent. The same solvent was used in the present study so that all measurements would be made under comparable conditions. While NMR measurements (Markley, 1975) indicate that acetic acid interacts with native RNase A and that high concentrations of acetic acid destabilize the protein [especially at a pH below 3.6 (Cann, 1971)], such destabilization does not occur at as low a concentration as 0.6%. This is also indicated by the fact that the thermal transition temperature of native RNase A at pH 5.0 is essentially unaffected by 0.6% acetic acid.

Temperature Dependence of ΔOD_{287}. The native and fully or partially regenerated RNase A (enzymatic activity in the range of 1.9–100%) were dissolved in degassed 0.6% acetic acid at a concentration of about 7.3×10^{-5} M, and the pH of the solution was adjusted to 4.00 (I = 0.015 M) or 5.00 (I = 0.064 M) with solid Tris base and negligible amounts of 1 M Tris base. The ultraviolet difference absorption at 287 nm (ΔOD_{287}) (Hermans & Scheraga, 1961) was measured for the protein solutions in a stoppered 1-cm silica cell with a Cary Model 14 spectrophotometer with a scale of 0.0–0.2. An empty *glass* cell (OD_{287} = 0.47) maintained at room temperature was used as a reference (instead of a protein solution) because the absorption of RNase A exhibits a small time-dependent change (see Results and Discussion), making it unsuitable as a reference. The instruments for temperature control and measurement were the same as those used in the measurement of the temperature dependence of the initial velocity. The temperature of each solution was increased from 10 to 80 °C in intervals of 2–4 °C and, at each temperature, 10–14 min was allowed for thermal equilibrium (Brandts & Hunt, 1967).

Temperature Dependence of $\Delta[\alpha]_{436}$. Native and fully or partially regenerated RNase A (enzymatic activity in the range of 1.9–100%) were dissolved in degassed 0.6% acetic acid at concentrations of (6.5×10^{-5})–(1.1×10^{-4}) M. The pH of the solution was adjusted to 4.00 (I = 0.015 M) as described in the previous paragraph. Difference optical rotation data were obtained at 436 nm with a Cary Model 60 spectropolarimeter, equipped with a 450 W xenon arc lamp. A water-jacketed quartz cell with a path length of 10 cm was used. The solvent base line was independent of temperature in the range from 10 to 80 °C. Hence, the base line was shifted off the chart to follow the small changes in $[\alpha]_{436}$, and the data are expressed[1] as $\Delta[\alpha]_{436}$ to distinguish them from the absolute values of the optical rotation. The temperature was controlled (to ±0.2 °C) by water flowing through the cell jacket with the use of a Haake Type F circulating bath. A calibrated thermistor probe was used to measure the temperature just beyond the cell. The temperature was increased for each solution from 10 to 80 °C in 2–4 °C intervals and, at each temperature, 10–14 min was allowed for thermal equilibrium (Brandts & Hunt, 1967).

Results and Discussion

Preparation of Partially Regenerated RNase A. Reshuffling of the disulfide bonds in the regeneration of RNase A can be arrested by lowering the pH of the solution from 8 to 4, since Schöberl & Gräfje (1958) and Eager & Savige (1963) observed that no disulfide interchange occurred spontaneously in the range of pH 1–6 with several disulfides related to cystine. This was verified for the protein as follows. At 70 min of regeneration, at which time the regain of enzymatic activity was 32%, the regeneration was stopped by lowering the pH of the solution to 4 with glacial acetic acid. The partially regenerated RNase A was isolated on Sephadex G-25 and was lyophilized by the same procedure used for the preparation of partially regenerated RNase A (see Experimental Section). Regeneration was restarted by dissolving the lyophilized protein in the reaction solution in which the concentrations of reduced and oxidized glutathione, EDTA, and Tris–acetate buffer were adjusted to be the same as those in the original reaction solution. The curve for the regain of enzymatic activity in the restarted solution as a function of reaction time was the same (within experimental error) as the original regain curve when the regeneration was not arrested. This result demonstrates that the regeneration process could be stopped by lowering the pH of the solution, and partially regenerated RNase A could be prepared without disturbing the disulfide bonds and the conformations of the proteins.

Temperature Dependence of the Initial Velocity at pH 5.00. The reproducibility of the absolute value of $\ln v_i$, where v_i is the initial velocity of hydrolysis of C>p by native and by fully or partially regenerated RNase A, was good only when the sample solution was not changed. This difference from one experiment to another may be due to variation of the slit width of the Cary 14, and this variation affects the initial velocity (Crook et al., 1960). The temperature dependence of $\ln v_i$, however, was relatively reproducible (because the slit width was fixed as the temperature was varied) and was analyzed separately for each RNase A solution.

The values of $\ln v_i$ increased linearly with the reciprocal of the absolute temperature as the temperature was increased up to ~37 °C, as shown in Figure 1. The initial velocity was almost constant from ~37 to ~53 °C, and decreased markedly above ~53 °C, as found by Matheson & Scheraga (1979).

The change at ~37 °C may reasonably be assumed to be due to an isomerization between an isomer (L), which is stable below ~37 °C, and an isomer (H), which is stable above ~37 °C. This assumption is supported by the fact that the enzymatic activity of isomer H, extrapolated to room temperature, is about 160% compared to that of isomer L, and such hyperactivity has been observed for the isomers of native RNase A (Walker et al., 1978; Watkins & Benz, 1978). The details of the conformations of these assumed isomers have been discussed by Matheson & Scheraga (1979). (Even if the assumption of such an isomerization were not correct, the following analysis would not be affected.)

Figure 1 shows that the thermal unfolding of isomer H in native and fully or partially regenerated RNase A starts above

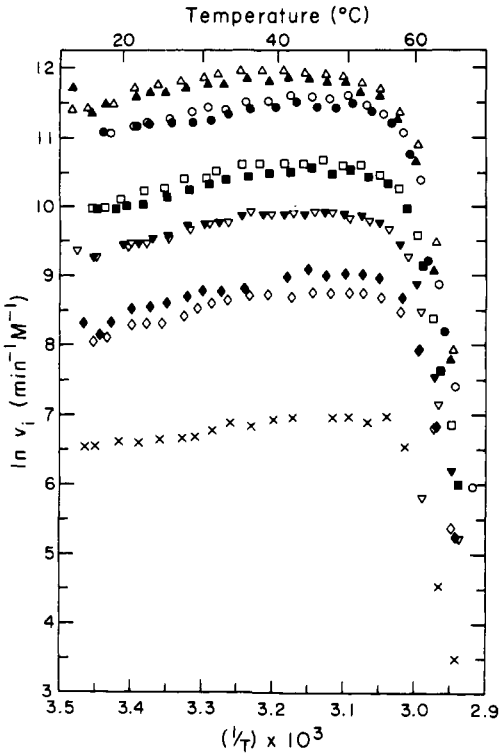

FIGURE 1: Arrhenius plot of the initial velocities (v_i) of hydrolysis of C>p by native and by fully or partially regenerated RNase A. The concentrations of the proteins were 3.3×10^{-7} (○) and 1.4×10^{-7} M (●) native RNase A, 3.2×10^{-7} (△) and 1.4×10^{-7} M (▲) fully regenerated RNase A, 9.2×10^{-7} (□) and 4.1×10^{-7} M (■) partially regenerated RNase A (enzymatic activity = 41%), 1.9×10^{-6} (▽) and 7.9×10^{-7} M (▼) partially regenerated RNase A (enzymatic activity = 18%), 6.0×10^{-6} (◇) and 2.6×10^{-6} M (◆) partially regenerated RNase A (enzymatic activity = 3.0%), and 2.2×10^{-5} M (×) partially regenerated RNase A (enzymatic activity = 0.6%). The concentration of the substrate, C>p, was 0.62 mM, and 0.6% acetate–Tris buffer (pH 5.00; I = 0.064 M) was used as the solvent.

48 °C. Thus, we extrapolated the apparent initial (constant) velocity for isomer H to temperatures above 48 °C to obtain the initial velocity of the thermally folded species in the temperature range of thermal unfolding (48–70 °C). The initial velocity of the thermally unfolded molecule was taken as zero, because the initial velocity of the native protein at 69.4 °C was only 0.34% of the extrapolated initial velocity of isomer H at 69.4 °C. The equilibrium constant K and the thermodynamic parameters ($\Delta H°$ and $\Delta S°$) for the transition from the thermally folded state to the thermally unfolded state (Schellman, 1955; Hermans & Scheraga, 1961) can be written as

$$K = \frac{[v_i]_H(T) - v_i(T)}{v_i(T) - [v_i]_U(T)} \quad (1)$$

$$\Delta H°(T_m) = -R[\mathrm{d} \ln K/\mathrm{d}(1/T)]_{T_m} \quad (2)$$

$$\Delta S°(T_m) = \Delta H°(T_m)/T_m \quad (3)$$

where $[v_i]_H(T)$ is the extrapolated initial velocity of isomer H at temperature T, $[v_i]_U(T)$ is the initial velocity of the thermally unfolded RNase A and is taken as zero at any temperature, $v_i(T)$ is the observed initial velocity at temperature T, and $\Delta H°(T_m)$ and $\Delta S°(T_m)$ are the enthalpy and entropy change, respectively, of thermal unfolding at the transition temperature, T_m. The logarithm of the equilibrium constant is plotted against the reciprocal of the absolute temperature in Figure 2. The line in Figure 2 was obtained from equilibrium thermal unfolding data for native RNase A (see

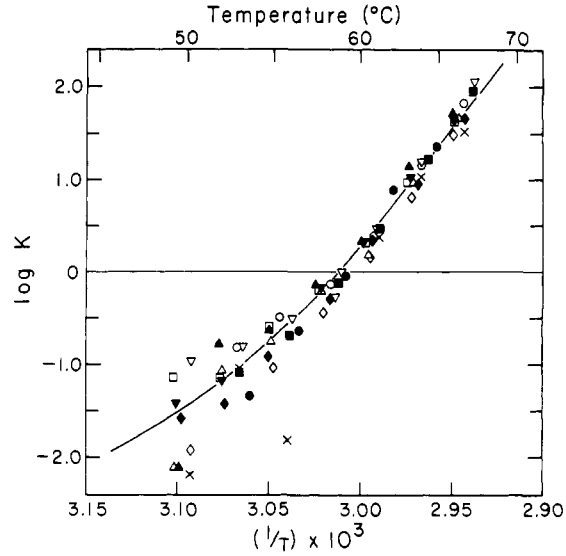

FIGURE 2: Logarithm of the equilibrium constant for the thermal transition, computed from the temperature dependence of the initial velocity at pH 5.00 by means of eq 1, is plotted against the reciprocal of the absolute temperature. The same symbols as those in Figure 1 are used to represent the data, obtained from Figure 1. The logarithm of the equilibrium constant computed from the temperature dependence of ΔOD_{287} for native and fully regenerated RNase A is shown as a solid line.

Table I: Melting Temperature and Thermodynamic Parameters of Native and Fully or Partially Regenerated RNase A at pH 5.00

enzymatic act.[a] (%)	transition temp (°C)	$\Delta H°$ (T_m) (kcal/mol)	$\Delta S°$ (T_m) (eu)	technique
native	59.4	119	358	initial velocity
native	59.0	104	314	ΔOD_{287}[c]
100[b]	58.8	100	301	initial velocity
100[b]	58.9	106	318	ΔOD_{287}[c]
41[b]	59.1	104	314	initial velocity
18[b]	58.9	102	306	initial velocity
3.0[b]	59.8	111	333	initial velocity
0.6[b]	59.8	111	333	initial velocity

[a] Enzymatic activity relative to that of native RNase A. [b] Fully or partially regenerated RNase A. [c] These results are reported under Temperature Dependence of ΔOD_{287} at pH 5.0.

Temperature Dependence of ΔOD_{287} at pH 5.00 under Results and Discussion). The *randomly distributed* deviations at low temperature (where K is very small) are simply due to the low accuracy of the data for the initial velocity (Crook et al., 1960). At higher temperatures, however, the data for the native or fully or partially regenerated RNase A are consistent with each other within the experimental errors. The melting temperature and the thermodynamic parameters at T_m are listed in Table I; within experimental error, these data are the same for all samples.

Since the thermodynamic profiles for the native and fully or partially regenerated RNase A (enzymatic activity in the range of 0.6–100%) are the same, we can classify the partially regenerated RNase A as a mixture of two types of components. One is the completely regenerated RNase which is enzymatically active and has the same thermodynamic behavior at the active site (including an assumed isomerization that occurs around 37 °C) as that of the native protein. The other includes all intermediates which do not take on an enzymatically active conformation in the temperature range from 10 to 70 °C.

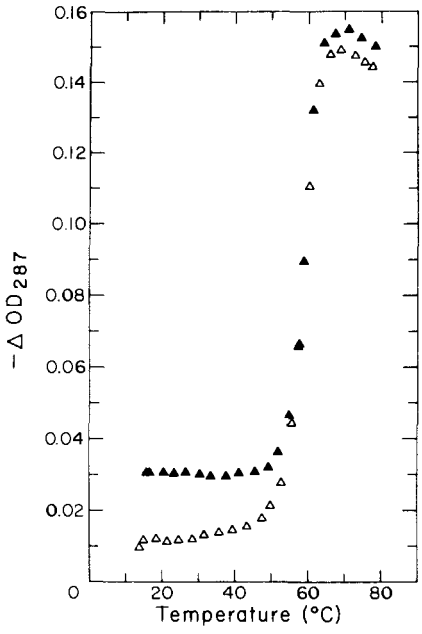

FIGURE 3: Temperature dependence of ΔOD_{287} for purified fully regenerated RNase A in 0.6% acetate–Tris buffer (pH 5.00; $I = 0.064$ M). Protein concentration is 7.4×10^{-5} M. The measurement was started about 1 (△) or 5 h (▲) after dissolving the protein at room temperature.

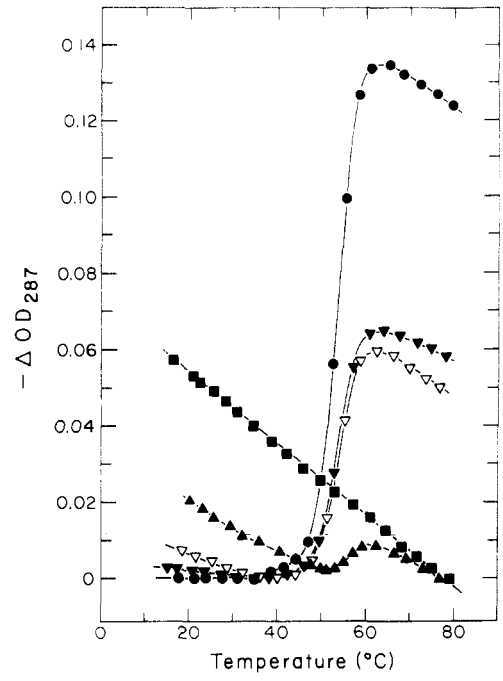

FIGURE 4: Temperature dependence of ΔOD_{287} (which was calculated by converting the concentration of protein in each case to 7.3×10^{-5} M) for native RNase A (●), partially regenerated RNase A (enzymatic activity = 13%) (▲), and partially regenerated RNase A (enzymatic activity = 1.9%) (■) in 0.6% acetic acid–Tris buffer (pH 4.00; $I = 0.015$ M). The time dependence of the curves is shown for a sample of partially regenerated RNase A (enzymatic activity = 52%); i.e., the measurement was started about 1 (▽) or 5 h (▼) after dissolving the protein. The smallest value of $-\Delta OD_{287}$ was arbitrarily taken as zero in each measurement.

Creighton (1979) reported that the intermediates have some enzymatic activity. Since it is unlikely that the thermodynamic parameters T_m, $\Delta H°(T_m)$, and $\Delta S°(T_m)$ would be the same for the active site of the intermediates and for that of the native protein (because of different disulfide pairings), we must attribute our observed values (which are identical for all partially regenerated material in the range of enzymatic activity of 0.6–100%) to *fully* regenerated RNase A. Thus, the enzymatic activity of the intermediates observed by Creighton (1979) may be due to contamination by fully regenerated RNase A which was not completely separated from the intermediates on his carboxymethylcellulose column. In fact, a long tailing can be observed in the elution curve of native RNase A on a carboxymethylcellulose column.

Temperature Dependence of ΔOD_{287} at pH 5.00. The thermodynamic profiles of native and purified *fully* regenerated RNase A were measured by observing the temperature dependence of ΔOD_{287} for comparison with the results of the measurements of initial velocity. Figure 3 shows the results for fully regenerated RNase A. One series of measurements (open triangles) was started about 1 h after dissolving the protein in 0.6% acetic acid; the same protein solution was also used for a series of measurements started after 5-h incubation at room temperature (solid triangles). The optical densities at 287 nm are different for the two curves at low temperature ($T < 48$ °C) and at high temperature ($T > 70$ °C), demonstrating that the thermally folded ($T < 48$ °C) and unfolded ($T > 70$ °C) conformations of fully regenerated RNase A at pH 5.00 are not uniform and show a slow time-dependent conformational change. The same time-dependent phenomena were observed for native RNase A. Such a slow time-dependent isomerization was also observed for native RNase A in a study of thin-film dialysis (Craig et al., 1963). Each melting curve was analyzed thermodynamically by using the method of Hermans & Scheraga (1961). Even though the thermally folded and unfolded conformations were different between the two curves, the character of the thermal unfolding, i.e., the difference in ΔOD_{287} between the thermally folded and unfolded states at the transition temperature, and $\Delta H°$ and $\Delta S°$ were independent of the isomerization.

The solid line in Figure 2 is a plot of the logarithm of the equilibrium constant against $1/T$. The thermodynamic parameters at the melting temperature were estimated, by using eq 2 and 3, and are listed in Table I. These thermodynamic properties are in good agreement with those of native and fully or partially regenerated RNase A computed from the temperature dependence of the initial velocity (Table I).

The fact that the thermodynamic parameters obtained from the optical density and the initial velocity measurements are the same demonstrates that the binding of the substrate, C>p, does not stabilize the conformation of the enzymatically active molecules and that the active site of the enzyme is thermally unfolded cooperatively with the environments of tyrosine residues.

Temperature Dependence of ΔOD_{287} at pH 4.0. Figure 4 shows the temperature dependence of ΔOD_{287} of native and of some of the partially regenerated RNase A samples (enzymatic activity = 1.9–52%) in 0.6% acetic acid–Tris buffer (pH 4.00; $I = 0.015$ M). Most of the molecules in the partially regenerated RNase A sample (enzymatic activity = 1.9%) were in disordered conformations because $-\Delta OD_{287}$ decreased linearly with increasing temperature and showed no thermal transition. The partially regenerated RNase A samples (enzymatic activity > 10%) showed a sharp increase of $-\Delta OD_{287}$ at around 54 °C which reflected the thermal unfolding of fully regenerated RNase A and of some ordered structures in the intermediates.

The values of ΔOD_{287} in the thermal transition region were measured in both directions (heating and cooling) and showed that the thermal transition is reversible below 61 °C, as previously observed for native RNase A (Hermans & Scheraga, 1961). If we extend the temperature up to 80 °C, some

irreversibility is observed, as in the case of native RNase A. The presence of some irreversibly denatured material at high temperature (>61 °C) slightly affects the curve for the temperature dependence of $-\Delta OD_{287}$ of the thermally unfolded molecules at high temperature, but such a small change has a negligible effect on the thermodynamic parameters and on the difference in $-\Delta OD_{287}$ between the thermally folded and unfolded states at the transition temperatures. Thus, the thermal transitions of the fully or partially regenerated RNase A were considered to be reversible and were analyzed in a similar way as for native RNase A (Hermans & Scheraga, 1961; Brandts & Hunt, 1967; Privalov et al., 1973).

The equilibrium constant K for the transition between thermally folded and unfolded states can be written as

$$K = \frac{(\Delta OD_{287})_F(T) - \Delta OD_{287}(T)}{\Delta OD_{287}(T) - (\Delta OD_{287})_U(T)} \quad (4)$$

where $(\Delta OD_{287})_F(T)$ is ΔOD_{287} of the thermally folded state extrapolated to temperature T, $(\Delta OD_{287})_U(T)$ is ΔOD_{287} of the thermally unfolded state extrapolated to temperature T, and $\Delta OD_{287}(T)$ is the measured value of ΔOD_{287} at the temperature T (Schellman, 1955). The thermodynamic parameters [$\Delta H°(T_m)$ and $\Delta S°(T_m)$] at the transition temperature can be calculated from eq 2 and 3.

$(\Delta OD_{287})_F(T)$ arises from two sources; i.e., one is the intermediates in disordered conformations, which shows a linear decrease of $-\Delta OD_{287}$ against temperature, as in Figure 4, and the other is the fully regenerated RNase A molecules and the ordered structure in the intermediates, for which $-\Delta OD_{287}$ could be assumed to decrease linearly with temperature in the *absence* of a thermal transition (Brandts & Hunt, 1967; Privalov et al., 1973). Thus, $-(\Delta OD_{287})_F(T)$ of the partially regenerated RNase A could be considered to decrease linearly with temperature from 10 to 80 °C. $(\Delta OD_{287})_U(T)$ similarly arises from two sources; i.e., one is the intermediates in thermally unfolded conformations, which shows a linear decrease of $-\Delta OD_{287}$ against temperature, and the other is the (high temperature) thermally unfolded regenerated RNase A molecules, for which $-\Delta OD_{287}$ could be assumed to decrease linearly with temperature, as for native RNase A (Hermans & Scheraga, 1961; Brandts & Hunt, 1967; Privalov et al., 1973). Thus, $-(\Delta OD_{287})_U(T)$ of the partially regenerated RNase A could also be considered to decrease linearly with temperature from 10 to 80 °C. $(\Delta OD_{287})_F(T)$ and $(\Delta OD_{287})_U(T)$ were evaluated in the regions where they changed linearly at low and high temperature (Figure 4) by least-squares curve fitting. As shown in Figures 3 and 4, $(\Delta OD_{287})_F(T)$ and $(\Delta OD_{287})_U(T)$ for partially regenerated RNase A (enzymatic activity = 52%) changed slowly with time. Hence, the measurements were repeated twice with the same stock RNase A solution (one being started about 1 h after dissolving RNase A and the other being started about 5 h after dissolving RNase A), and the analyses were performed separately for each curve.

The fraction of thermally unfolded molecules in RNase A was evaluated from the expression for K of eq 4 and is plotted against temperature for the partially regenerated RNase A (enzymatic activity = 52%) in Figure 5. The two curves for the partially regenerated RNase A in Figure 4 become coincident by excluding the temperature and time dependence of $(\Delta OD_{287})_F(T)$ and $(\Delta OD_{287})_U(T)$ for each transition curve separately. The difference between $(\Delta OD_{287})_F(T)$ and $(\Delta OD_{287})_U(T)$ at the transition temperature, T_m, was also reproducible, i.e., 0.0671 and 0.0659 for the curves of Figure 4 plotted with the symbols (▽) and (▼), respectively. Thus,

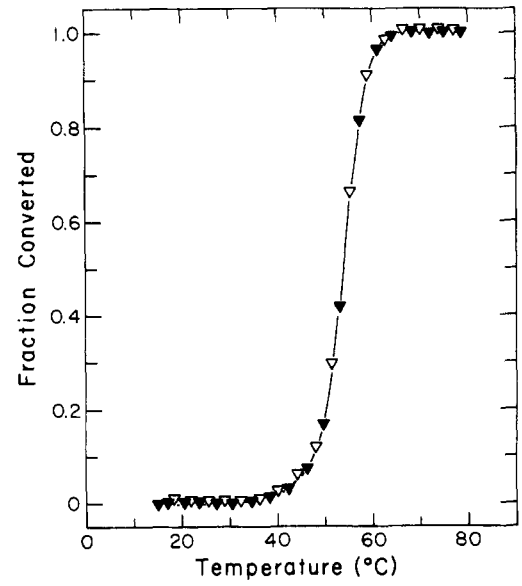

FIGURE 5: Fraction of thermally unfolded molecules in partially regenerated RNase A (enzymatic activity = 52%). Measurements were started about 1 (▽) or 5 h (▼) after dissolving the protein.

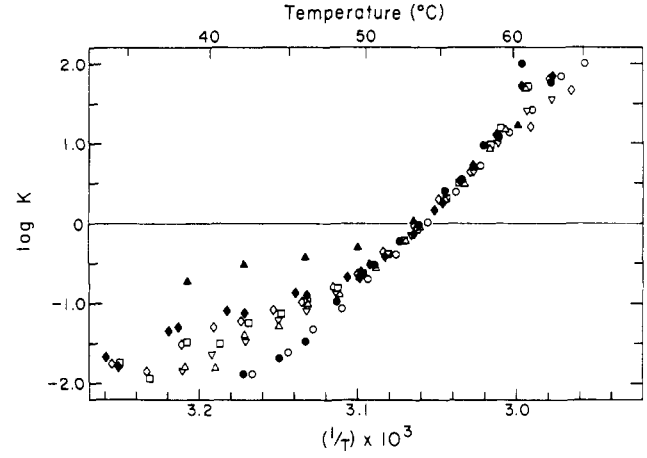

FIGURE 6: Logarithm of the equilibrium constant for the thermal transition, calculated from the temperature dependence of ΔOD_{287} at pH 4.00, is plotted against the reciprocal of the absolute temperature. The data are the results of duplicate measurements (i.e., at 1 and 5 h) for each RNase A solution as shown in Figure 3. The concentrations of protein were 7.9×10^{-5} M native RNase A (●), 7.1×10^{-5} M fully regenerated RNase A (○), 8.1×10^{-5} M partially regenerated RNase A (enzymatic activity = 92%) (△), 7.5×10^{-5} M partially regenerated RNase A (enzymatic activity = 72%) (□), 9.4×10^{-5} M partially regenerated RNase A (enzymatic activity = 52%) (▽), 1.04×10^{-4} M partially regenerated RNase A (enzymatic activity = 39%) (◇), 1.07×10^{-4} M partially regenerated RNase A (enzymatic activity = 25%) (◆), and 1.11×10^{-4} M partially regenerated RNase A (enzymatic activity = 13%) (▲).

the difference between the two curves for the partially regenerated RNase A (enzymatic activity = 52%) in Figure 4 is attributed merely to the time dependences of $(\Delta OD_{287})_F(T)$ and $(\Delta OD_{287})_U(T)$. For native and fully or partially regenerated RNase A (enzymatic activity in the range of 10–100%), similar results were obtained, and the character of the thermal unfolding was reproducible for any partially regenerated RNase A by excluding the temperature and time dependence of $(\Delta OD_{287})_F(T)$ and $(\Delta OD_{287})_U(T)$ in the same manner as described above.

The logarithm of K is plotted against the reciprocal of the absolute temperature for native and fully and partially regenerated RNase A (enzymatic activity in the range of

Table II: Transition Temperature and Thermodynamic Parameters for Native and Fully or Partially Regenerated RNase A at pH 4.00

enzymatic act.[a] (%)	transition temp[b] (°C)	ΔH° (T_m) (kcal/mol)	ΔS° (T_m) (eu)
native[c]	53.5	90	274
100	54.0	95	289
92	53.7	88	271
72	53.7	88	270
52	53.8	85	259
39	53.4	78	240
25	53.7	86	262
13	53.0	61	186
native[c]	53.1	94	287
100	53.2	95	290
92	53.3	85	259
60	53.6	87	266
41	53.2	91	279
25	54.2	103	316
13[d]	54.6	129	394
13[d]	53.9	86	264

[a] Enzymatic activity relative to that of native RNase A. [b] The first eight lines were obtained from measurements of ΔOD_{287}, and the second eight lines were obtained from measurements of $\Delta[\alpha]_{436}$. [c] Native RNase A; all other entries pertain to fully or partially regenerated RNase A. [d] Since the thermodynamic parameters were not consistent between the two melting curves (after 1 and 5 h, respectively, of dissolving the protein), both of them are listed.

10–100%) in Figure 6. The curves of log K vs. $1/T$ are similar for native and for fully and partially regenerated RNase A (enzymatic activity > 25%) around the thermal transition temperature. The thermodynamic parameters for the thermal unfolding of the proteins were calculated from Figure 6 by using eq 2 and 3 at the thermal transition temperatures. The results are listed in Table II. The thermal transition temperatures and the values of ΔH° and ΔS° at the transition temperature are the same for native RNase A and for fully and partially regenerated RNase A (enzymatic activity > 25%), within experimental error. The partially regenerated RNase A, however, showed larger values of log K than that of native or fully regenerated RNase A below 50 °C. This indicates that some fraction of the intermediates has ordered structures which unfold thermally at lower temperature (T_m ~ 40 °C) than the thermal unfolding (T_m = 54 °C) of the fully regenerated molecules at pH 4. It is difficult to estimate the experimental error in K at low temperature. The fact, however, that the low-temperature data for the fully regenerated material agree with those for native RNase A and that the corresponding data for *all* partially regenerated material lie above the curve for native RNase [rather than scattering about it, as do the data of Figure 2] suggests that our evidence for the existence of ordered structures in the intermediates is reasonably well established (beyond experimental error). The large deviation of the melting curve of partially regenerated RNase A (enzymatic activity = 13%) from that of native RNase A might arise from the low accuracy of the results for this sample because of the small change of ΔOD_{287} induced by the thermal unfolding (see Figure 4) or might indicate the presence of ordered structure in the intermediates. The indicated ordered structure in the intermediates may play an important role, as a nucleation site(s), in the regeneration process.

In Figure 7, the values of $-(\Delta OD_{287})_{F-U}$, which is the difference between $(\Delta OD_{287})_F$ and $(\Delta OD_{287})_U$ at the melting temperature and reflects the amount of regenerated molecules and of intermediates having ordered structure (in the partially regenerated RNase A), are plotted against the enzymatic

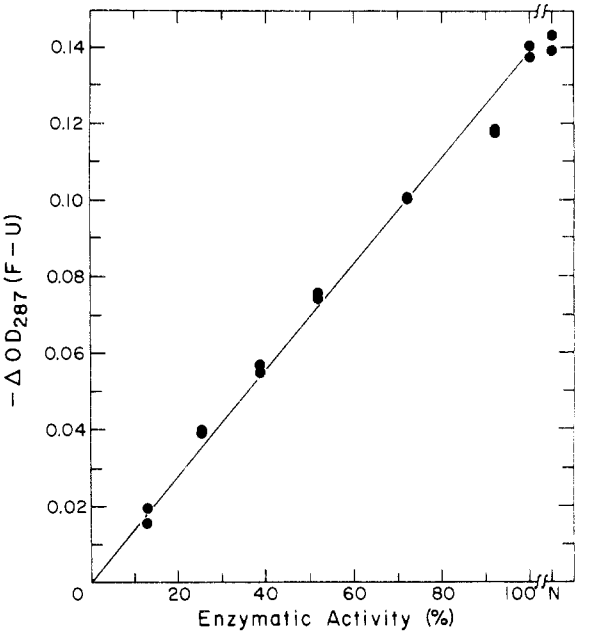

FIGURE 7: Differences in ΔOD_{287} between thermally folded and unfolded states $[(\Delta OD_{287})_{F-U}]$ at the transition temperature for native and fully or partially regenerated RNase A at pH 4.00 are plotted against their enzymatic activities. "N" designates native RNase A. The points at 100% enzymatic activity were obtained with purified regenerated material, whereas those at "N" were for the original native protein.

activity of the partially regenerated RNase A (which corresponds quantitatively to the amount of fully regenerated molecules). The increase of $-(\Delta OD_{287})_{F-U}$ is linear with enzymatic activity. This demonstrates that the dominant conformations of the intermediates are disordered and that the amount of the intermediates having ordered structure is small, even though they might exist (because of the deviations at low temperature in Figure 6).

Temperature Dependence of $\Delta[\alpha]_{436}$ *at pH 4.0.* Since the variation of $\Delta[\alpha]_{436}$ reflects primarily conformational changes in the backbone of a protein, the temperature dependence of $\Delta[\alpha]_{436}$ was recorded for native and fully or partially regenerated RNase A (enzymatic activity in the range of 1.9–100%) in 0.6% acetic acid–Tris buffer (pH 4.00; I = 0.015 M). Some of the results are shown in Figure 8.

Since the results are similar to those for ΔOD_{287} vs. temperature in Figure 4, the same thermodynamic analysis was performed as that in Figures 4–6, except that ΔOD_{287} was changed to $\Delta[\alpha]_{436}$ in eq 4. The logarithm of the equilibrium constants, K, is plotted against the reciprocal of the absolute temperature for native RNase A and fully or partially regenerated RNase A (enzymatic activity in the range of 10–100%) in Figure 9. The curves of log K vs. $1/T$ for partially regenerated RNase A (enzymatic activity in the range of 10–100%) are similar to that for native or fully regenerated RNase A above the thermal transition temperature. As in Figure 6, intermediates, whose thermal transition temperature is ~40 °C, might be the origin of the large deviations at low temperature, as discussed for the ΔOD_{287} measurements. Table II lists the thermodynamic parameters which were calculated for the thermal unfolding of the proteins from Figure 9 by using eq 2 and 3. The results agreed with those obtained in the ΔOD_{287} measurements (Table II). Similar to Figure 7, the value of $(\Delta[\alpha]_{436})_{F-U}$, which is the difference between $(\Delta[\alpha]_{436})_F$ and $(\Delta[\alpha]_{436})_U$ at the melting temperature and reflects the amount of regenerated molecules and of intermediates having ordered structure (in the partially regen-

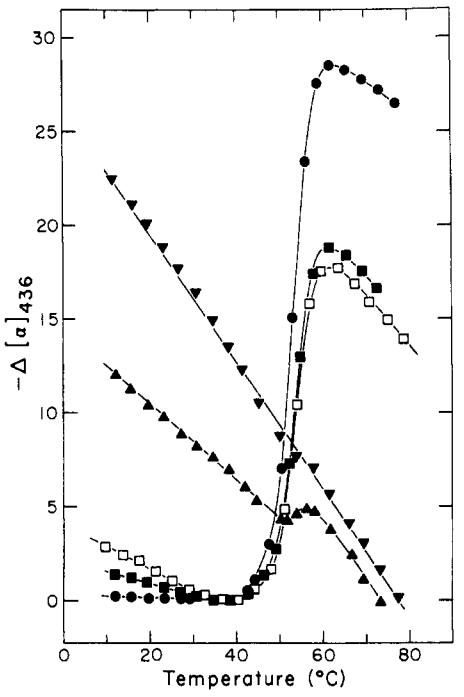

FIGURE 8: Temperature dependence of $\Delta[\alpha]_{436}$ in deg cm^2/(g dm) for native RNase A (●), partially regenerated RNase A (enzymatic activity = 13%) (▲), and partially regenerated RNase A (enzymatic activity = 1.9%) (▼) in 0.6% acetic acid–Tris buffer (pH 4.00; I = 0.015 M). The time dependence of the curves is shown for a sample of partially regenerated RNase A (enzymatic activity = 72%); i.e., the measurement was started about 1 (□) or 5 h (■) after dissolving the protein. The smallest value of $-\Delta[\alpha]_{436}$ was arbitrarily taken as zero for each curve.

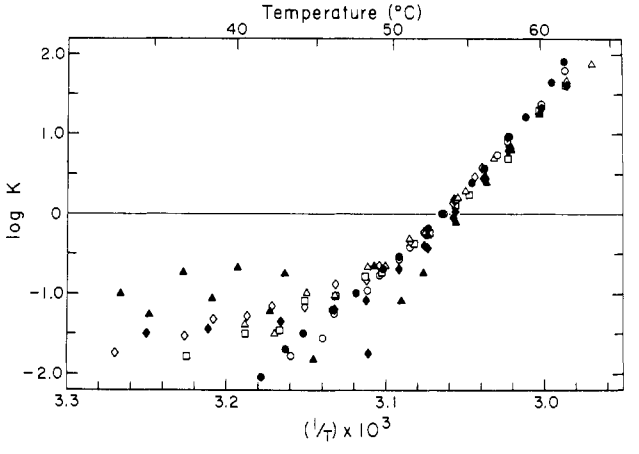

FIGURE 9: Logarithm of the equilibrium constant for the thermal transition, calculated from the temperature dependence of $\Delta[\alpha]_{436}$ at pH 4.00, is plotted against the reciprocal of the absolute temperature. The data are the results of duplicate measurements (i.e., at 1 and 5 h) for each RNase A solution. The concentrations of protein were 7.0×10^{-5} M native RNase A (●), 7.5×10^{-5} M fully regenerated RNase A (○), 6.3×10^{-5} M partially regenerated RNase A (enzymatic activity = 92%) (△), 6.8×10^{-5} M partially regenerated RNase A (enzymatic activity = 72%) (□), 7.8×10^{-5} M partially regenerated RNase A (enzymatic activity = 41%) (◇), 8.3×10^{-5} M partially regenerated RNase A (enzymatic activity = 25%) (◆), and 9.7×10^{-5} M partially regenerated RNase A (enzymatic activity = 13%) (▲).

erated RNase A), increased linearly with enzymatic activity; i.e., the extent to which the backbone structures are regenerated is the same as that to which the active site is regenerated. This also demonstrates that the dominant conformations of the intermediates are disordered and that the amount of the intermediates having ordered structures is small, even though they might exist (because of the deviations at low temperature in Figure 9).

Conclusion

In conclusion, the intermediates are enzymatically inactive and their dominant conformations are disordered. Small fractions of the intermediates appear to have ordered structures, which might take part in the regeneration process as nucleation sites.

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Regeneration of Ribonuclease A from the Reduced Protein. 2. Conformational Analysis of the Intermediates by Nuclear Magnetic Resonance Spectroscopy†

Yasuo Konishi and Harold A. Scheraga*

ABSTRACT: Bovine pancreatic ribonuclease A was regenerated from the reduced protein by glutathione in the same way as described in the first paper of this series [Konishi, Y., & Scheraga, H. A. (1980) *Biochemistry* (preceding paper in this issue)]. The ^{1}H NMR spectrum of reduced ribonuclease A was typical of that of a disordered conformation. Since the intermediates in the early stages of the regeneration process exhibited a similar NMR spectrum in the aromatic region (but with less splitting of each resonance) as that of reduced ribonuclease A, the dominant conformations of the intermediates are more disordered. At all stages of the regeneration process, the resonance of each histidine residue [except His-12 C(2)] appeared as only two peaks, one corresponding to a disordered environment of the histidine residue and the other to that of native ribonuclease A. The resonances of the regenerated His-12 C(2) proton (which were also present in the spectrum of the reduced protein and in that of native ribonuclease A) disappeared in the spectrum of partially regenerated ribonuclease A, demonstrating a local conformational difference between regenerated and native ribonuclease A. It appears that the His-12 residue exists in two different environments in regenerated ribonuclease A (both being enzymatically active), and the slow interconversion between them broadens the resonance so that it disappears. The areas of the resonances of the regenerated His-48 C(2), His-105 C(2) and C(4), and His-119 C(2) protons in partially regenerated ribonuclease A were consistent with the degree of regeneration determined in the previous paper of this series. The area of the resonance of all His-C(2) protons corresponding to the disordered environment was also consistent with the degree of regeneration. Thus, the NMR data demonstrate that the dominant conformations of the intermediates are disordered.

The nuclear magnetic resonance (NMR)[1] technique has been used to study the folding of bovine pancreatic ribonuclease A (RNase A) (Matthews & Westmoreland, 1973; Benz & Roberts, 1975a,b). The resonances of His-12 C(2)-H, His-48 C(2)-H, His-105 C(2)-H and C(4)-H, and His-119 C(2)-H in native RNase A and of His C(2)-H in denatured RNase A can be resolved and have recently been reassigned (Bradbury & Teh, 1975; Markley, 1975a; Patel et al., 1975; Shindo et al., 1976). The chemical shifts and areas of these resonances provide two types of information. The chemical shift provides information as to whether a given residue is in the native, denatured, or some other state (Zaborsky & Millman, 1972; Blum et al., 1978), while the area of a resonance is proportional to the relative amount of a given conformation.

With this information, NMR studies have supported two models of the folding process. One is the two-state model proposed by Schellman (1955). Two-state behavior was observed in the thermal unfolding in D_2O at *high* pD (above pD 4.4); i.e., an identical decrease was observed in the areas of the four His C(2)-H resonances of the folded form with increasing temperature (Matthews & Westmoreland, 1973; Benz & Roberts, 1975a); similarly, no local unfolding of the environments of the aromatic residues was observed below the temperature of thermal unfolding (Lenstra et al., 1979). In the modification introduced by Chavez & Scheraga (1980a) into the unfolding pathway proposed by Burgess & Scheraga (1975), which pertains to unfolding near neutral pH in H_2O, intermediates are involved, with His-12 and -48 unfolding before His-105 and -119. The other model involves a stepwise unfolding. This behavior was observed in the equilibrium thermal unfolding in D_2O at *low* pD (below pD 3.4) (Matthews & Westmoreland, 1973; Benz & Roberts, 1975a) or in urea or guanidine hydrochloride unfolding (Benz & Roberts, 1975b); i.e., the environments of His-12 and His-119 *unfold* before the environments of His-48 and His-105. In a kinetic

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[1] Abbreviations used: NMR, nuclear magnetic resonance; RNase A, bovine pancreatic ribonuclease A; His C(2)-H and His C(4)-H, the C(2) and C(4) protons of histidine, respectively; 2'-CMP, cytidine 2'-monophosphoric acid; DSS, 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt; UV, ultraviolet; ORD, optical rotatory dispersion; ΔOD_{287}, difference optical density at 287 nm; $\Delta[\alpha]_{436}$, optical rotation at 436 nm minus a constant value to bring it on scale; pD, pH of D_2O solution corrected with the relation pD = pH meter reading + 0.40 (Glasoe & Long, 1960); Tyr ϕ(2,6)-H, the ring protons at C(2) and C(6) of tyrosine; Tyr ϕ-(3,5)-H, the ring protons at C(3) and C(5) of tyrosine; Phe ϕ-H, the ring protons of phenylalanine; $(EA)_{rel}$, enzymatic activity of regenerated RNase A relative to that of native RNase A.

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