Study of the Thermal Denaturation of Ribonuclease A by Differential Thermal Analysis and Susceptibility to Proteolysis

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1. The thermally induced change in conformation of ribonuclease A in solution was investigated by differential thermal analysis and the susceptibility of the enzyme to proteolytic digestion by ficin. 2. A transition with a mid-point of 60.5°C at pH 4.2 was observed directly by differential thermal analysis and shown to be a property of the native structure. 3. At pH 4.2 ribonuclease A is susceptible to ficin digestion at 60°C but not at 18°C. 4. Chromatographic analysis of the digestion products reveals that transient active intermediates are produced during the digestion. 5. Three of these intermediates were purified and partially characterized. 6. The nature of those sections of the ribonuclease molecule that are involved in the thermal transition is discussed.

In aqueous solution bovine pancreatic ribonuclease undergoes a pH-dependent thermal transition, which produces changes in its physical parameters, enzymic activity and susceptibility to proteolytic digestion (for reviews see Scheraga & Rupley, 1962; Ooi, Rupley & Scheraga, 1963). These changes can be used to monitor the denaturation process, which is reversible under certain conditions. The thermal transition has also been studied by direct calorimetry with an adiabatic solution calorimeter (Kreshek & Scheraga, 1966) and two different types of differential thermal analysers (Beck, Gill & Downing, 1965; Danforth, Krakauer & Sturtevant, 1967). This paper is in part concerned with the further investigation by DTA† of the thermal denaturation of ribonuclease in solution. Although this technique provides direct evidence for a thermal transition, it does not demonstrate that a change in the tertiary structure has occurred during the transition. Native ribonuclease is only susceptible to digestion by proteolytic enzymes of a very wide specificity, e.g. pronase (Kesner, Muntwyler & Griffin, 1964), whereas thermally denatured ribonuclease is susceptible to digestion by two highly specific endopeptidases, trypsin and chymotrypsin (Rupley & Scheraga, 1963; Ooi *et al.* 1963). This increase in susceptibility is attributed to a change in the conformation of ribonuclease associated with the thermal transition. Ficin, an endopeptidase of

plant origin with a wide specificity towards polypeptide substrates, cannot digest native ribonuclease but it can digest the thermally denatured enzyme. The digestion of ribonuclease A by ficin has been studied under conditions comparable with those used in the DTA experiments to investigate whether the transitions observed by DTA are associated with changes in the conformation of ribonuclease.

MATERIALS AND METHODS

Enzymes. Ribonuclease A was prepared from 4× recrystallized bovine pancreatic ribonuclease (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.) by ion-exchange chromatography on either Amberlite IRC-50 (British Drug Houses Ltd., Poole, Dorset, U.K.) and the procedure of Hirs, Moore Stein (1953) or CM-cellulose (Whatman CM-52) as described by Taborsky (1959). The material was then freed of low-molecular-weight components by gel filtration on Sephadex G-25 (Pharmacia Ltd., Uppsala, Sweden) in 0.05M-ammonium acetate, which was subsequently removed by freeze-drying. The homogeneity of these preparations was checked by analytical ion-exchange chromatography on the appropriate medium and by starch-gel electrophoresis as described below. The freeze-dried material was stored in a desiccator over P₂O₅.

Ficin was obtained from dried fig latex (Koch-Light Laboratories Ltd.) by the method of Hammond & Gutfreund (1959). The $V_{\max}$ of this preparation towards *N*-benzoyl-L-arginine ethyl ester was of the order 6 μ mol hydrolysed/min per mg dry wt. of ficin at 25°C. During this preparation all solutions were made up from O₂-free water and stored under an atmosphere of O₂-free N₂. This preparation was further purified by chromatography on

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† Abbreviation: DTA, differential thermal analysis.

CM-cellulose (Whatman CM-52) in the presence of EDTA. A NaCl gradient running from 0.05 M to 0.4 M in mM-EDTA, pH 6.0, was employed to fractionate the material. Fractions of constant specific activity from the component present in the greatest amount were bulked and stored under a N₂ atmosphere in 10 mM-cysteine-HCl, pH 6.0. Under these conditions activity could be preserved for 2-3 weeks. Freeze-drying caused irreversible inactivation.

Enzyme assays. Ribonuclease activity was usually determined by the spectrophotometric method of Crook, Mathias & Rabin (1960b), by using cytidine 2':3'-phosphate (potassium salt, monohydrate) synthesized by the method of Crook, Mathias & Rabin (1960a). The purity of the substrate was checked by t.l.c. on polyethyleneimine cellulose with 0.25 M-NaCl as the solvent (Randerath, 1962). Uridine 2':3'-phosphate (barium salt) (Sigma Chemical Co., St Louis, Mo., U.S.A.) was also employed as a substrate in the spectrophotometric assay procedure of Richards (1955). The rate of hydrolysis of RNA was determined as described by Kunitz (1946). Yeast RNA (sodium salt; British Drug Houses Ltd.), was subjected to gel filtration on Sephadex G-25 to remove low-molecular-weight components and freeze-dried. Buffers were made up from tris (Koch-Light Laboratories Ltd.), which had been recrystallized from hot methanol-water (12:5, v/v).

Ficin preparations were assayed by following the hydrolysis of benzoylarginine ethyl ester spectrophotometrically (Holloway, Mathias & Rabin, 1964). The ester was lot AR4 from Yeda Research and Development Co., Rehovoth, Israel and was chromatographically pure.

A Cary model 14 recording spectrophotometer was used in the spectrophotometric assays.

Differential thermal analysis. The experiments described in this paper were performed on the Du Pont 900 Differential Thermal Analyser (Du Pont Co. Ltd., London E.C.4, U.K.). The sample and reference (small glass beads) were placed in small glass phials (0.3 cm × 2.7 cm) in cavities in the heating block, which was heated from room temperature at the rate of 10°C/min. Initially the sample consisted of 0.04 ml of a 23% (w/v) solution of ribonuclease in sodium acetate buffer, pH 4.2 (I.0.1). Subsequently, however, 0.025 ml of a 10% solution was found to be adequate. The speed of heating was chosen carefully because if it was too slow the system reached equilibrium and small transitions were not observed, and if it was too fast the lag in the recording system caused the transition to be observed at a higher temperature. Urea (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.) was recrystallized from ethanol and dried in the air on a pump. Fresh solutions were always employed.

Proteolytic digestions. Digestions were performed under anaerobic conditions to minimize the inactivation of ficin through oxidation of the thiol group, which is essential for enzymic activity. All solutions were made up from O₂-free deionized water and the digestions were performed under an atmosphere of O₂-free N₂. Typically a digestion mixture consisted of 5 mg of ribonuclease A (final concn. 0.146 mM), 1.25 ml of sodium acetate buffer, pH 4.2 (I.0.1), 0.5 ml of 2 M-NaCl soln., 0.25 ml of mM-cysteine (adjusted to pH 4.2 with HCl), 0.25 ml of 10 mM-EDTA and 0.25 ml of deionized water. The reaction vessel, which was stoppered to prevent concentration changes through evaporation, was placed in a constant-temperature water bath. Digestion was initiated by the

addition of 0.05 ml of ficin solution (approx. 7.5 μM), which was obtained by diluting the stock preparation 1:100. If this dilution was made with mM-cysteine, cysteine could be omitted from the digestion mixture under anaerobic conditions. Digestion was terminated by the addition of 20 mM-iodoacetamide soln. to a final concentration of 1 mM. This rapidly inactivated the ficin by carboxymethylation of the essential thiol group. Iodoacetamide was shown to inhibit neither the catalytic activity of native ribonuclease nor the renaturation of thermally denatured ribonuclease. In the preparative-scale experiments, the concentrations of all components were increased proportionately.

To follow the course of a digestion samples (0.05 ml) were removed from the digestion mixture at suitable time intervals and pipetted into 4.95 ml of 0.1 M-tris-HCl buffer, pH 7.15 (I.0.2), containing mM-iodoacetamide. The extent of digestion was then determined by assaying 2 ml of this solution, corresponding to 40 μg of the original ribonuclease, against cytidine 2':3'-phosphate at 25°C. The iodoacetamide (British Drug Houses Ltd.) was recrystallized twice from aq. 50% (w/v) ethanol and the crystals were dried *in vacuo* over P₂O₅ and stored away from the light.

Chromatographic analysis of the digestion mixtures. Digestion mixtures up to 10 ml in volume were loaded directly on to a Sephadex G-25 column (20 cm × 1.6 cm diam.), which had been poured and equilibrated in 0.05 M-ammonium acetate, pH 6.35. Preparative-scale digestions were freeze-dried and redissolved in a suitable volume of the ammonium acetate and analysed on a larger column (40 cm × 2.5 cm diam.). The effluent was monitored by measuring *E*₂₈₀ and conductivity. Conductivity measurements were made with a Radiometer conductivity meter, type CDM 2d (Radiometer, Copenhagen, Denmark). The deionized protein fractions, which were excluded from the Sephadex matrix, were bulked and freeze-dried. In preparative run III only, a Sephadex G-50 column (50 cm × 2.5 cm diam.) was used instead of Sephadex G-25 to obtain the protein fraction. The protein fractions were subsequently fractionated on a column (20 cm × 0.9 cm diam.) of CM-cellulose (Whatman CM-52), which had been equilibrated in 5 mM-tris-HCl buffer, pH 8.0. Material was eluted from the column with an exponential gradient (0-0.15 M-NaCl) in the tris buffer (250 ml mixing vessel). The protein content, conductivity and ribonuclease activity of each effluent fraction was determined. In the preparative-scale experiments a larger column (25 cm × 1.4 cm diam.) and mixing vessel (1 litre) were employed. Fractions eluted from the CM-cellulose, corresponding to an active intermediate, were bulked, freeze-dried, deionized on Sephadex G-25 and freeze-dried again. This material was then chromatographed again on a CM-cellulose column (20 cm × 0.9 cm) equilibrated in 5 mM-tris-HCl buffer, pH 8.0, with a 0.01-0.1 M-NaCl gradient and the active fragment was isolated as in the previous step. The homogeneity of an active fragment was checked by starch-gel electrophoresis or by chromatography.

Starch-gel electrophoresis. Starch gels were prepared with partially hydrolysed starch (Connaught Medical Research Laboratories, Toronto, Canada) by the method of Smithies (1955). The discontinuous buffer system of Poulik (1957) was employed and electrophoresis was

carried out for 4 h at 100 V and a current of 20–25 mA. Protein was detected in the gels by staining with Amido Black 12B (G. T. Gurr Ltd., London S.W.6, U.K.)

Amino acid analyses. The sample of salt-free, freeze-dried protein was hydrolysed in 6 M-HCl in a sealed evacuated tube for 22 h at $110 \pm 0.5^\circ\text{C}$. The hydrolysates were analysed on a Technicon amino acid AutoAnalyzer. Many samples were oxidized with performic acid before hydrolysis (Hirs, 1956). Correction factors for the loss of tyrosine, threonine, serine, methionine and cystine, which occurs during hydrolysis, were calculated from data obtained by hydrolysing three identical samples of ribonuclease A for 22, 48 and 72 h respectively. The maintenance of the column temperature at 60°C throughout the run and the loading of the sample in 0.01 M-HCl instead of 0.1 M-HCl allowed complete resolution of the methionine sulphone, aspartic acid, threonine and serine complex by using the automatic twin-column procedure of Hackett, Mathias & Pennington (1965).

Ultraviolet-absorption spectra. A Cary model 14 recording spectrophotometer was used to obtain the spectra, which were measured in 0.1 M-tris-HCl buffer, pH 7.15 (I.0.2), at 25°C in a 1 cm path-length cell.

Optical-rotatory-dispersion spectra. A Polarmatic 62 recording spectropolarimeter (Bellingham and Stanley Ltd., London, N.15, U.K.), which was connected to an X-Y plotter and fitted with a thermostatically controlled cell compartment (25°C), was used to measure these spectra over the range 235–435 nm. The sample, 0.6 ml of a $50 \mu\text{M}$ solution of enzyme in 0.1 M-tris-HCl buffer, pH 7.0 (I.0.02), was placed in a 1 cm path-length cell and N_2 was flushed through the system to permit measurements at lower wavelengths.

N-terminal-group determination. A modified form of the fluorodinitrobenzene technique of Sanger (1949) was employed. Reaction between fluorodinitrobenzene and the proteins was carried out in *N*-ethylmorpholine acetate buffer, pH 8.5, and the DNP protein was separated from low-molecular-weight reaction products by gel filtration on Sephadex G-25 in 0.1 M- NH_3 (Ambler, 1963). The solvent systems of Brenner, Niederwieser & Pataki (1965) were used to identify the DNP-amino acids on silica gel thin-layer plates.

RESULTS AND DISCUSSION

Differential thermal analysis. Fig. 1 shows that at pH 4.2 ribonuclease undergoes a thermal transition, which commences at 53°C (onset temperature), and is completed by 72°C (thermogram A). The mid-point of this transition, T_{tr} , occurs at 60.5°C (after correction of the chromel-alumel thermocouple temperature). This compares with a value of 58°C for T_{tr} based on changes in physical parameters at pH 4.2 (Hermans & Scheraga, 1961). The reversibility of this transition was investigated by again running the sample used in thermogram A after it had been allowed to cool to room temperature (thermogram B). T_{tr} and the shape of the endothermic peak are unchanged but the depth of the peak has been decreased by 25%. If a solution of ribonuclease is maintained at a temperature

Fig. 1. Differential thermal analysis of ribonuclease. All the analyses except G and H were performed in acetate buffer (I.0.1). The thermograms are displaced vertically for ease of comparison and each division on the ordinate scale is 0.2°C . A, 0.04 ml of 23% solution of ribonuclease, pH 4.2; B, re-run of A; C, 0.025 ml of 10% solution of ribonuclease, pH 4.2; D, sodium acetate buffer, pH 4.2 (I.0.1); E, 0.025 ml of 11.5% solution of oxidized ribonuclease A, pH 4.2; F, 0.025 ml of 20% solution of ribonuclease in 8 M-urea-sodium acetate buffer, pH 4.2 (----, control, 8 M-urea-0.1 M-sodium acetate buffer, pH 4.2); G, 0.025 ml of 10% solution of ribonuclease in tris-HCl buffer, pH 7.0 (I.0.1); H, 0.025 ml of 10% solution of ribonuclease in 25% (v/v) propan-1-ol-0.1 M-tris buffer, pH 7.0; I, 0.04 ml of 10% solution of ribonuclease in 0.01 M-sodium acetate buffer (pH 5.5)-0.1 M-sodium sulphate; J, 0.02 ml of 20% solution of ribonuclease in 0.01 M-sodium acetate buffer, pH 5.5, plus 0.02 ml of 0.04 M-cytidine 2'(3')-phosphate (equimolar), pH 5.5 (nucleotide/enzyme ratio 5.5) (----, nucleotides only); K, 0.02 ml of 20% solution of ribonuclease, pH 5.5, plus 0.02 ml of 0.01 M-cytidine 2'(3')-phosphate, pH 5.5 (nucleotide/enzyme ratio 1.37); L, 0.04 ml of 23% solution of ribonuclease, pH 4.2, plus 0.04 ml of 7.5 mM-cytidine 3'-phosphate (nucleotide/enzyme ratio 0.45).

above the transition temperature for a long period, irreversible denaturation is known to occur. This probably accounts for the difference between thermograms A and B. If the original sample was allowed only to reach 70 – 80°C in the first run and then cooled rapidly, irreversible denaturation was minimized and the transition was almost completely reversible. Thermogram C, which was obtained with 0.25 ml of a 10% solution of ribonuclease, suggests that T_{tr} is independent of the concentration

of the protein. The depth of the peak is decreased by 45%. A control experiment in which the buffer alone was analysed (thermogram D) shows that the observed transition is not due to the buffer system nor is it an artifact of the instrument. Kreshek & Scheraga (1966) have reported that at 45°C a slow exothermic transition occurs in concentrated (i.e. 1%) solutions but not in more dilute (0.1%) solutions. Although very concentrated solutions of ribonuclease were used in the DTA experiments, this exothermic transition would not be expected to be observed or to affect the value of T_{tr} because of the rapid rate of heating employed.

The absence of a transition in oxidized ribonuclease A (thermogram E) and in ribonuclease A in 8M-urea (thermogram F) at pH 4.2 over the temperature range investigated, reveals that the endothermic process is associated with structural features of the native conformation of ribonuclease. Similarly, a sharp transition is not observed in the physical properties of oxidized and urea-denatured ribonuclease under these conditions. Therefore, it is suggested that the transition observed by DTA in the native enzyme is the same as the one shown by changes in physical parameters.

Thermograms G and H show that rupture of hydrophobic bonding is probably involved in the denaturation process. The presence of 25% (w/v) propan-1-ol lowers T_{tr} from 62°C to 52.5°C. The propan-1-ol would lower the pK of a cationic buffer only very slightly. However, around neutral pH values, T_{tr} is independent of pH and has a maximum value of 63°C. Therefore the lowering of T_{tr} must be due to the effect of the propan-1-ol on hydrophobic interactions. A T_{tr} of 50°C has been calculated for ribonuclease in 10% (w/w) propan-1-ol at pH 6.5–6.8 from changes in E_{287} (Schrier & Scheraga, 1962).

The interaction of certain anions, e.g. sulphate (Ginsburg & Carroll, 1965) and nucleotides (Nelson, Hummel, Swenson & Friedman, 1962) with ribonuclease has been shown to stabilize the native conformation. Thermogram I shows that, at pH 5.5, 0.1M-sodium sulphate raises T_{tr} from 62°C to 65°C. Approx. 93% of the enzyme was calculated to have bound sulphate under these conditions. A re-run of this sample gave an identical value of T_{tr} , showing that the sulphate anions did not interfere with the renaturation process. The interaction of the substrate analogues, cytidine 2'- and 3'-phosphate, at the active site is greatest at pH 5.5 and therefore analyses were performed at this pH rather than at pH 4.2. Thermogram J shows that the formation of a 1:1 nucleotide-enzyme complex raises the transition temperature to 71.5°C. That the stabilization results from the formation of nucleotide-enzyme complex is demonstrated in thermograms K (T_{tr} 68.5°C) and L (T_{tr} 61.5°C).

Fig. 2. Calculation of energy of activation from DTA thermogram. When the extent of the reaction lies between 0.05 and 0.8 then ΔT is related to the absolute temperature, T , by the following equation:

$$\ln \Delta T = C' - E/RT$$

where C' is a constant. The plots A, B, C and H correspond to thermograms A, B, C and H in Fig. 1.

In these samples approx. 95% and 37.5% respectively of the enzyme are calculated to have bound nucleotides at 25°C. The control experiments indicate that the nucleotides alone do not contribute to the endothermic transition.

The energy of activation, E , of a chemical reaction can be calculated from a DTA thermogram by a method devised by Piloyan, Ryabchirov & Novikova (1966) (see Fig. 2). The results in Table 1 show that the energy of activation for the thermal denaturation of ribonuclease is independent of the concentration of the ribonuclease. However, partial irreversible denaturation causes an apparent rise in E . The lowering of E to 36.0 kcal/mol at pH 7.0 in the presence of 25% (w/v) propan-1-ol is consistent with a decrease in T_{tr} due to the disruption of hydrophobic bonding. The formation of a nucleotide-enzyme complex raises E to 114.5 kcal/mol.

Digestion of ribonuclease A by ficin. No loss of ribonuclease activity was observed when ribonuclease was incubated with ficin at 25°C and pH 4.2 in the digestion mixture described in the Materials and Methods section. However, on performing the digestion at 60°C and pH 4.2,

Table 1. *Energy of activation for thermal denaturation of ribonuclease calculated from DTA thermograms*

The thermograms are those listed in Fig. 1.

Thermogram	Sample	<i>E</i> (kcal/mol)
A	Ribonuclease A (0.04 ml of 23% soln., pH 4.2)	78.5
B	Re-run of A	96.0
C	Ribonuclease A (0.025 ml of 10% soln., pH 4.2)	79.0
H	Ribonuclease (0.025 ml of 10% soln. in 25% propan-1-ol-0.1M-tris buffer, pH 7.0)	36.0

conditions under which ribonuclease is known to undergo a reversible thermal transition, virtually all the ribonuclease activity was destroyed within 10 min. No loss of ribonuclease activity was detected when the digestion mixture was maintained at 60°C and pH 4.2 in the absence of ficin. The temperature-dependence of the loss of activity (Fig. 3) shows that ribonuclease activity is destroyed only at temperatures at which ribonuclease is known to be at least partially thermally denatured at pH 4.2. Thus susceptibility to proteolytic digestion by ficin is a sensitive way of monitoring thermally induced changes in the conformation of ribonuclease.

In considering factors that affect the digestion of ribonuclease by ficin it is important to differentiate between those that influence the activity of the ficin and those that affect the conformation of the substrate ribonuclease. Fig. 4, which summarizes the progress of digestion under various conditions, shows that controlled proteolysis is only obtained under anaerobic conditions. This is because ficin is rapidly inactivated in an aerobic system despite the presence of cysteine. If more ficin is added to the digestion after all the original ficin has been inactivated then a further decrease in the ribonuclease activity is recorded. The ability of ribonuclease A to renature successfully is not affected by maintaining it at 60°C in the presence of mM-cysteine and oxygen. Increasing the ficin/ribonuclease ratio accelerated the loss of activity, confirming that proteolysis was responsible. The complete loss of all the ribonuclease activity demonstrated that all the ribonuclease was denatured into a form susceptible to ficin proteolysis. The demonstration that ribonuclease is susceptible to proteolytic digestion by ficin at pH 4.2 and 60°C suggests that the thermal transition observed by DTA is associated with a change in the conformation of ribonuclease.

DTA had shown that the presence of 2'-oxygen

Fig. 3. Effect of temperature on the digestion of ribonuclease by ficin. Digestions were performed for 10 min at each temperature and then the loss of activity was determined as described in the Materials and Methods section. The digestion mixture consisted of: 5 mg of ribonuclease A; 1.25 ml of sodium acetate buffer, pH 4.2 (10.1); 0.5 ml of 2M-NaCl; 0.25 ml of mM-cysteine, pH 4.2; 0.25 ml of 10mM-EDTA and 0.25 ml of deionized water. A ficin preparation (approx. 7.5 μ M; 0.05 ml) was used in each digestion. The ribonuclease/ficin ratio was 1000:1.

Fig. 4. Digestion of ribonuclease by ficin under aerobic and anaerobic conditions. The digestion mixture was as for Fig. 3. The temperature was 60°C and pH 4.2. Samples (0.05 ml) were removed for the determination of ribonuclease activity. $\blacktriangle$, 7.5 μ M-ficin, aerobic; $\bullet$, 7.5 μ M-ficin, anaerobic; $\blacksquare$, 68.5 μ M-ficin, anaerobic; ----, no ficin.

pyrimidine nucleotides affected the thermal transition of ribonuclease. Therefore two parallel digestions were performed in the absence and presence of a mixture (mM) of cytidine 2'- and 3'-phosphate (nucleotide/enzyme ratio 6.85). The initial rate of digestion was identical in both experiments. Thus the number of susceptible bonds whose cleavage prevents the successful renaturation of ribonuclease is not decreased by the presence of the nucleotides. Two factors probably account for this. First, ficin

has a very wide specificity and an analysis of the digestion products suggests that ficin proteolysis does occur simultaneously at several points in thermally denatured ribonuclease. Many of these may be remote from the binding sites for the nucleotides. Secondly, under the experimental conditions approx. 50% of the enzyme would be bound to nucleotide at 25°C and this would increase T_{tr} by a few degrees. However, at 60°C a significant proportion of the ribonuclease would have undergone the thermal transition.

In a following section it will be demonstrated that digestion by ficin results in transient intermediates possessing enzyme activity. The existence of such intermediates confirms that some of the initial points of proteolysis are not involved in the active site. Therefore the binding of the nucleotide at the active site need not affect the thermal unfolding of other parts of the molecule. It does, however, affect the change in conformation associated with the changes in physical parameters. Thus ficin proteolysis could lead to the loss of ability to renature before ribonuclease underwent the complete thermal transition associated with changes in physical parameters.

Markus, Barnard, Castellani & Saunders (1968) have observed that cytidine 2'- and 3'-phosphate protect ribonuclease against inactivation by trypsin and chymotrypsin at pH 5.5 and 60°C. They have postulated that the binding of nucleotide causes changes in the conformation distant from the active site, thereby affecting the proteolytic susceptibility.

Further information about the course of the digestion was obtained by analysing the progress curve of an anaerobic digestion in the form of a semi-logarithmic plot. Fig. 5 shows that there are

Fig. 5. Time-course analysis of anaerobic digestion of ribonuclease by ficin at 60°C and pH 4.2. The digestion mixture was as for Fig. 3. Samples (0.05 ml) were removed for the determination of ribonuclease activity.

three distinct phases in the digestion. Initially, there is a rapid loss of activity, which is first-order with respect to ribonuclease. The first-order rate constant for this process, k , is $6.42 \times 10^{-3} \text{ s}^{-1}$. An intermediate period follows in which the rate constant changes as the faster inactivating processes go to completion. Finally there is the slow loss of residual activity, which probably reflects the digestion of a relatively stable intermediate, which is capable of renaturation to a derivative retaining 7% activity. The first-order rate constant for this slowest step leading to complete inactivation is $1.67 \times 10^{-4} \text{ s}^{-1}$. Thus this analysis suggests that the course of digestion is complex and that intermediates are produced that can renature to an active derivative of ribonuclease. The rate of proteolysis was followed by measuring the production of ninhydrin-positive material as a function of time (Fig. 6). This showed that proteolysis continued after the loss of all the ribonuclease activity and revealed that the preliminary intermediates were susceptible to further digestion. No increase in ninhydrin colour resulted from incubating the digestion mixture at 60°C in the absence of ficin.

Chromatographic analysis of the digestion products by a combination of gel filtration and

Fig. 6. Evidence of proteolysis during digestion of ribonuclease by ficin. The digestion was carried out in a Radiometer pH-stat at 60°C and pH 4.2. The digestion mixture consisted of 4.7 mg of ribonuclease, 1.25 ml of mM-cysteine, 0.25 ml of 10 mM-EDTA, 0.5 ml of O_2 -free deionized water, 0.5 ml of 2 M-NaCl and 0.3 ml of a 750 μM ficin solution. Ninhydrin colour value was determined by adding a sample (0.1 ml) from the digestion mixture to 9.9 ml of 0.1 M-tris-HCl buffer, pH 7.0. This was diluted 20-fold and then analysed on a Technicon AutoAnalyzer set up to measure ninhydrin-positive material. ●, % increase in ninhydrin colour; ■, % of ribonuclease activity remaining.

ion-exchange chromatography also provided information about the course of the digestion. Digests that had been halted at various degrees of completion were first passed through a column of Sephadex G-25. This resolved the digestion into two fractions, the protein fraction containing intact ribonuclease and the larger digestion products, and the peptide fraction containing low-molecular-weight products and constituents of the digestion mixture (Fig. 7). The appearance of tyrosine-containing material in the latter confirmed that proteolysis had occurred. Ribonuclease activity was confined to the protein fraction, which was bulked and freeze-dried without loss of activity.

The analysis of the protein fractions by ion-exchange chromatography on CM-cellulose revealed that the loss of ribonuclease activity during digestion was due to the fragmentation of thermally-denatured ribonuclease into a complex mixture of polypeptides (Fig. 8). Comparison of the various elution patterns showed that throughout most of the digestion, ribonuclease activity was found in several components, e.g. B, C and D in Fig. 8. As the extent of the digestion increased, the proportion of these transient active intermediates decreased and there was an accumulation of inactive digestion products that were only slightly retarded by the column, e.g. E in Fig. 8. The chromatographic behaviour of the active intermediates suggested that only very limited proteolytic modification of ribonuclease had occurred in their production.

Several peptide fractions were also analysed either on CM-cellulose under comparable conditions or on Sephadex G-10. They all contained a heterogeneous mixture of tyrosine-containing peptides, even those derived from the early stages of the digestion. This indicated that the initial attack probably led to the further destabilization of the protein conformation and the exposure of more susceptible bonds. This rapid secondary proteolysis would account for the production of small peptides. However, at some points the initial attack must be faster than the subsequent proteolysis as relatively stable high-molecular-weight intermediates capable of renaturation to active derivatives of ribonuclease are also produced early in the digestion. This last situation is comparable with the formation of ribonuclease S (Richards, 1955). Thus the digestion of thermally denatured ribonuclease is complex and must proceed by more than one pathway simultaneously.

Isolation and partial characterization of some active digestion intermediates. As the nature of these transient active intermediates could indicate which sections of ribonuclease are involved in the thermal transition three preparative-scale digestions were performed. The scaling-up of the experiment was necessary as the yields of these fragments were very

Fig. 7. Gel filtration of digestion mixture, halted after 59% of the ribonuclease activity had been destroyed. A sample (10 ml) was loaded directly on to a column (20 cm $\times$ 1.6 cm diam.) of Sephadex G-25 and eluted with 0.05 M-ammonium acetate. Fractions (2 ml) were collected. —, E_{280} ; ----, conductivity at 18°C.

Fig. 8. Chromatography of protein fraction on CM-cellulose. The sample (400 mg) was derived from a digestion in which 55% of the ribonuclease activity had been destroyed. Fractionation was achieved with a 0–0.15 M-NaCl exponential gradient in 5 mM-tris-HCl buffer, pH 8.0. Fractions (3 ml) were collected. —, E_{280} ; ····, ribonuclease activity (arbitrary units); ----, conductivity. Native ribonuclease A elutes as a single component corresponding to peak A.

low (Table 2). The chromatographic analyses of these digestions were all similar and an active intermediate, peak C in run II (Fig. 8) appeared in a corresponding position in all the elution patterns. This was selected for further investigation and was isolated and purified by rechromatography on CM-cellulose and Sephadex G-25. Although the yield of active fragment from run I was sufficient only for an amino acid analysis, the yields from runs II and III were sufficient for several other experiments designed to investigate the catalytic activity and structure of the active fragments.

Table 3 shows that the amino acid compositions of the three purified fragments differ only very slightly, if at all, from that of ribonuclease A. This suggests that to obtain an active fragment proteolytic digestion must not exceed the cleavage of one or two peptide bonds or the removal of a short

Table 2. *Summary of preparative digestions*

The active fragments (e.g. peak C in Fig. 8) were isolated by a combination of gel filtration and ion-exchange chromatography as described above.

Digestion	Wt. of ribonuclease digested (mg)	Extent of digestion (%)	Yield of active fragment	
			mg	% of starting material
I	500	28	4	0.8
II	500	55	14	2.8
III	1500	60	17	1.1

Table 3. *Amino acid composition of ribonuclease A and the active fragments*

Active fragments were prepared as described in Table 2. Molar ratios were calculated on the norm of alanine = 12 residues. Th., theoretical composition. Ox., oxidized samples. Cystine and methionine were determined as cysteic acid and methionine sulphone for oxidized samples.

Amino acid	Ribonuclease A			Active fragment		
	Th.	Ox.	Native	I	II	III
Asp	15	14.9	15.61	14.81	15.21	15.55
Thr	10	10.2	10.82	10.15	9.98	9.89
Ser	15	15.5	15.15	14.24	15.1	13.76
Glu	12	11.1	12.44	13.59	11.45	11.28
Pro	4	3.54	5.06	4.20	3.63	3.78
Gly	3	3.17	3.07	3.18	3.26	3.18
Ala	12	12	12	12	12	12
Val	9	9.14	8.89	8.72	8.71	9.12
I						
Cys	8	7.39	7.68	7.46	7.95	7.91
Met	4	3.56	3.96	3.72	3.82	3.61
Ile	3	2.78	2.76	2.79	2.66	2.94
Leu	2	2.12	2.08	2.11	2.07	2.04
Tyr	6	5.55	6.22	5.83	5.83	5.97
Phe	3	3.12	3.09	3.1	3.04	3.04
Lys	10	10.27	10.78	10.18	9.88	10.2
His	4	4.08	4.18	3.85	3.85	4.15
Arg	4	4.05	4.12	4.01	3.96	4.12
Total	124	122.47	127.9	123.94	122.55	122.54
No. of analyses	—	4	4	4	2	4

segment of the polypeptide chain. Further proteolysis must cause extensive disruption of the conformation of thermally denatured ribonuclease and destroy its ability to renature to an active conformation. *N*-Terminal-group analyses showed that fragment II possessed lysine and histidine *N*-terminal groups whereas fragment III possessed lysine and arginine *N*-terminal groups. The lysine group was assumed to be lysine-1 as a glutamic end group was not detected and the amino acid analyses did not suggest the loss of a lysine residue. The appearance of a new *N*-terminal group in these derivatives eliminated the possibility that they were conformational isomers of ribonuclease A produced on renaturation. Knowledge of the amino acid composition and *N*-terminal groups is

not sufficient to permit an unequivocal identification of the point of proteolytic cleavage. However, the new *N*-terminal histidine in fragment II could be histidine-105, which is accessible according to X-ray data (Kantha, Bello & Harker, 1967) and is preceded by a lysine residue. Peptide or ester bonds involving the carboxyl group of lysine in synthetic substrates are very susceptible to hydrolysis by ficin. Formation of fragment III appears to involve the loss of a serine residue and the production of a new *N*-terminal arginine residue. Arginine-33 is preceded by a serine residue and is in a section of the polypeptide chain, residues 26–33, thought to be involved in the thermal transition (Ooi *et al.* 1963). Thus proteolysis of thermally denatured ribonuclease could be initiated at both these points, i.e. Lys–His

Table 4. *Catalytic activity of fragments*

V for fragments was measured relative to that for ribonuclease A.

	Substrate			
	Cytidine 2':3'-phosphate		Uridine 2':3'-phosphate RNA	
	$10^3 \times K_m$	V	$10^3 \times K_m$	V
Ribonuclease A	0.83	1.0	1.03	1.0
Fragment II	1.87	0.46	—	0.23
Fragment III	0.81	1.05	1.11	1.0

(104–105) and Ser–Arg (32–33), confirming that the digestion was complex.

Table 4 records the enzymic activities of the fragments and ribonuclease A. Although the arrangement of the groups in the active site must be essentially the same in the fragments and ribonuclease A, small differences in their tertiary structure could account for the differences in activity. The active intermediates in the tryptic digest of ribonuclease were found to have a decreased activity (Ooi *et al.* 1963). In contrast ribonuclease in which two of the disulphide bridges have been cleaved chemically hydrolyses cytidine 2':3'-phosphate more rapidly than does the native enzyme (Neumann, Steinberg, Brown, Goldberger & Sela, 1968). Thus the activity results are consistent with fragments with a slightly altered conformation.

There are maxima in the u.v.-absorption spectra of the fragments at 277.5 nm, the same wavelength as in ribonuclease A. On a weight basis ribonuclease A and the fragments have the same extinction at this wavelength. The ratios of the extinction at 260 nm to 280 nm and at $\lambda_{\max}$ to $\lambda_{\min}$ are different for each fragment and different from those for ribonuclease A. This again suggests that the conformations of the fragments are slightly different from that of the parent enzyme. However, the optical-rotatory-dispersion spectra of both fragments showed a Cotton effect centred at 280 nm. In the native enzyme this effect has been attributed to the asymmetric environment of the three abnormally ionizing tyrosine residues, which are involved in tyrosyl-carboxyl interactions essential for the maintenance of the active conformation. Thus the environment of these tyrosine residues appears to be unaltered in the fragments. Although two out of three abnormal tyrosine residues are normalized by thermal denaturation those parts of the polypeptide chain involved in these interactions do not appear to be the points of initial attack by ficin.

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