Ribonuclease E, an Intermediate in the Degradation of Ribonuclease by Porcine Elastase

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Bovine pancreatic ribonuclease has been studied as the substrate of a number of proteolytic enzymes. In the native state it is completely resistant to trypsin and chymotrypsin (1, 2), but denaturation by chemical (3) or physical (4, 5) means induces susceptibility to these enzymes. Ribonuclease is, however, susceptible to attack by a number of other proteases. Subtilisin has been shown to cause extensive degradation of the molecule (6), with the formation of an enzymically active intermediate which can, under controlled conditions, be isolated in almost quantitative yield (7). Pepsin will also cause extensive degradation. Either active (8) or inactive (9) intermediates may be formed, but only the inactive intermediate has been isolated. Although ribonuclease is hydrolyzed only very slowly by carboxypeptidase (10), it has been observed that impure carboxypeptidase preparations can cause extensive degradation of ribonuclease without a concomitant loss of enzymic activity (11, 12). The identity of the contaminating activities was not determined. However, since neither trypsin nor chymotrypsin degrades native ribonuclease, the results with impure carboxypeptidase suggest the existence of a third pancreatic protease which can degrade native ribonuclease.

Elastase has only recently come to be recognized as a distinct member of the family of pancreatic proteolytic enzymes (13). Its specificity has been found to be very broad and different from that of either trypsin or chymotrypsin in that elastase exhibits a relative preference for peptide bonds involving the carboxyl group of aliphatic amino acids (14). Relatively little attention has been paid to the action of elastase on native globular proteins, although its unusual specificity lends interest to such studies. The results of the investigation to be reported in this paper show that elastase is capable of extensively degrading native ribonuclease. The course of the proteolysis offers some unusual features, notably an initial delay, and the accumulation of active intermediates.

EXPERIMENTAL PROCEDURE

Materials—The ribonuclease used was Sigma Chemical Company, type III, of assorted lots, each of which was chromatographed before use by the method of Hirs, Stein, and Moore (15), desalted as described by Richards and Vithayathil (7), and lyophilized. Crystalline porcine pancreatic elastase was obtained from the Worthington Biochemicals Corporation, Lots 5658 and 5659. Crystalline, salt-free trypsin was also a Worthington product. Diisopropyl fluorophosphate was obtained from Mann Research Laboratories. Carboxymethyl cellulose was Serva Lot 02119 containing 0.56 meq per g. Amberlite XE-64 was obtained from the Rohm and Haas Company, and was sized and washed by the method of Hirs, Stein, and Moore (15).

Ribonuclease assays were performed with RNA as the substrate as previously described (10), or titrimetrically in the pH-stat with the barium salt of cytidine 2',3'-cyclic phosphate as substrate. A solution, 3 ml, containing 3 mg per ml of the cyclic phosphate in 0.3 M NaCl is placed in the pH-stat maintained at 25° and brought to pH 7 with 0.02 N NaOH. The initial rate of uptake of 0.02 N NaOH after the addition of ribonuclease is then measured. The pH is maintained at 7.0. This rate is linear at least up to 10 μg of ribonuclease A.

The pH-stat used consisted of a Radiometer type TTTit titrator and Radiometer SBR 2b titrigraph together with a type of SBU la syringe burette, also obtained from Radiometer. A jacketed beaker, through the walls of which water was circulated from a constant temperature bath, served as the reaction vessel. Stirring was accomplished with a small magnetic stirring bar. Contamination with atmospheric CO2 was controlled with a continuous stream of CO2-free N2, saturated with water vapor at the appropriate temperature, which was passed over the surface of the reaction mixture. The rate of nitrogen flow could generally be adjusted so that negligible base uptake was recorded in the blank reactions. Alkali uptake at pH 8 was converted to bonds broken per mole of the substrate. The pH-stat main vessel. Stirring was accomplished with a small magnetic stirring bar. Contamination with atmospheric CO2 was controlled with a continuous stream of CO2-free N2, saturated with water vapor at the appropriate temperature, which was passed over the surface of the reaction mixture. The rate of nitrogen flow could generally be adjusted so that negligible base uptake was recorded in the blank reactions. Alkali uptake at pH 8 was converted to bonds broken per mole of the substrate. The pH-stat main vessel. Alkali uptake at pH 8 was converted to bonds broken per mole of the substrate. The pH-stat main vessel.
dinitrobenzene technique of Sanger (18) as modified by Levy (19). Separation of the ether-soluble 2,4-dinitrophenylamino acids was performed by paper chromatography with the two-dimensional system recommended by Levy (19) or the tertiary amyl alcohol system of Blackburn and Lowther (20); the latter system was also used for examining the aqueous phase.

Performic acid oxidation was performed as described by Hirs (21).

Amino acid analysis was carried out with the automated system of Spackman, Stein, and Moore (22) by Analytica Corporation of New York. The samples were hydrolyzed in constant boiling HCl for 24 hours prior to analysis. Ninhydrin determinations of free amino groups were performed by the method of Moore and Stein (23). Protein concentrations were estimated by absorption measurements at 280 mn or 215 mn in the Beckman model DU spectrophotometer.

RESULTS

Fig. 1 is taken from the pH-stat record of a typical digestion of RNase A§ by elastase and describes the time course of the reaction. It will be noticed that more than 20 bonds are broken per molecule of ribonuclease when the reaction processes completion. The fact that approximately one out of every six peptide bonds in ribonuclease is susceptible to hydrolysis indicates an extraordinary lack of specificity on the part of elastase, comparable indeed to that of subtilisin (6). The lag period seen in the pH-stat record is typical of a large number of digests and is a feature of the elastase-catalyzed breakdown of ribonuclease which has not commonly been observed with other proteolytic systems when protein is the substrate.

A study of the elastase digestion of ribonuclease at three different pH values showed that the characteristic delay is present at pH values as high as 9.4 (Fig. 2). It is seen, however, that the lag becomes less marked as the over-all rate of the reaction is increased. A similar phenomenon has been noted on increasing the temperature at constant pH. The data shown in Fig. 2 contradict the possibility that the lag is due to an initial reaction in which an amino group of abnormally high pK§ is liberated. This possibility is effectively eliminated by the observation that the lag is present when either the increase of ninhydrin reactive material or the change in absorption at 285 mn is used as a measure of the course of the reaction.

In view of the known heterogeneity of crystalline elastase, the possibility was considered that the observed kinetics may be the result of the consecutive action of two separate enzymes. Accordingly, the enzyme preparation was chromatographed on a carboxymethyl cellulose column essentially according to the procedure of Naughton and Sanger (14). This procedure resolved the elastase preparation into three components, only one of which displayed substantial proteolytic activity. It was found that equivalent results are obtained regardless of whether the chromatographically purified or the crystalline material is used. It is likely then that the lag must be explained on the basis of the action of a single enzyme on ribonuclease.

Residual ribonuclease activity, as a function of the average number of peptide bonds broken by elastase, is shown in Fig. 3. The shape of the curve is typical of that of a number of digests carried out under similar conditions. It has generally been found that breakage of the first few bonds by elastase results in a smaller activity loss than does cleavage of subsequent bonds. It is to be noted that a large number of bonds must be broken before ribonuclease activity is reduced to half the initial level.

Data of the type shown in Fig. 3 suggest the possible presence of active intermediates in the digestion mixture since a small number of peptide bonds are hydrolyzed without appreciable loss of activity. Evidence for the existence of active intermediates was, therefore, sought by chromatographic analysis of elastase digests of ribonuclease.

Chromatography of the digest was carried out on columns of Amberlite XE-64 as described by Hirs, Stein, and Moore (15). The starting ribonuclease used gives a single sharp peak on the column, in a position corresponding to ribonuclease A. Elution analysis of elastase digests demonstrated the presence of a new,
and in similar experiments was isolated as a salt-free lyophilized powder by procedures similar to those described by Richards and Vithayathil (7) except that carboxymethyl cellulose was substituted for Amberlite XE-64. RNase E prepared in this manner has catalytic properties which are very similar to those of RNase A. Fig. 5 shows that the activities of the two proteins, when assayed for their ability to convert ribonucleic acid into acid-soluble material, are identical. In Fig. 6, the two proteins are compared with the 2',3'-cyclic phosphate of cytidylic acid as the substrate. Their activities are again seen to be essentially the same, however, in their susceptibility to tryptic inactivation as shown in Table I. It is seen that while RNase A activity is essentially unchanged by incubation with trypsin at 37° for 1 hour, the activity of RNase E is completely destroyed under the same conditions.

A first insight into the nature of the bonds broken in the conversion of RNase A to RNase E came as the result of an examination of the amino-terminal residues of the modified enzyme by the fluorodinitrobenzene technique. RNase A is a single polypeptide chain with lysine at its amino end (24). RNase E was found to contain NH₂-terminal serine as well as NH₂-terminal lysine. It can therefore be concluded that RNase E is composed of two polypeptide chains, held together by either disulfide bonds (5) or by noncovalent forces or both (7).

Since RNase S, the active intermediate produced in the subtilisin digestion of RNase A, also contains only NH₂-terminal lysine and serine, and since the chromatographic, enzymatic, and stability properties of RNase E are very similar to those of RNase S (25), the possibility was entertained that RNase E is very similar to or perhaps even identical with RNase S.

The molecule of RNase S is composed of two polypeptide chains and retains many of the properties of RNase A. The 20-residue peptide at the NH₂ terminus does not dissociate under ordinary conditions even though it is no longer covalently attached to the rest of the molecule. RNase S may, however, be resolved into S-peptide and S-protein by careful treatment with trichloroacetic acid (25). Neither component of RNase S is by itself capable of hydrolyzing RNA while a stoichiometric mixture regains, almost fully, the original activity. The experiment presented in Fig. 7 shows that RNase E is also resolved by trichloroacetic acid into two inactive fractions which, upon recombination in a 1:1 molar ratio also exhibit, almost fully, the activity of the original protein. It is clear on the basis of this experiment that RNase E
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FIG. 5. A comparison of the ability of RNase A and RNase E to convert ribonucleic acid into acid-soluble material at pH 5 and 25°.

FIG. 6. A comparison of the rates at which RNase A and RNase E hydrolyze the low molecular weight substrate cytidine 2',3'-cyclic phosphate. The pH was 7.0 and the temperature 25°. Further details are given in the text.

behaves in a manner identical to that of RNase S in this highly specific test.

In order to characterize RNase E more precisely, the material was subjected to quantitative amino acid analysis along with a sample of E-peptide (the material present in the supernatant fluid after trichloroacetic acid treatment of RNase E) and of E-protein (which is the trichloroacetic acid-precipitable portion of RNase E). Table II shows the results of these analyses together with literature values for the amino acid compositions of RNase A (or S) and of S-peptide and S-protein. The amino acid compositions shown for RNase E and RNase S, and their derivatives, are experimental numbers which have not been corrected for hydrolytic or other losses. The values shown for RNase A are, on the other hand, idealized numbers (3). The table shows that, while the amino acid composition of E-protein is identical to that of S-protein, E-peptide contains 1.5 fewer residues of alanine than does S-peptide. In all other respects, however, the compositions of E-peptide and of S peptide are identical. A comparison of RNase E and of RNase S also shows only a difference of between 1 and 2 alanine residues per molecule. Since there are 2 alanine residues at the carboxyl terminus of S-peptide (Residues 19 and 20 of ribonuclease) it may be

TABLE I

Effect of trypsin on activity of RNase A and RNase E

Experimental details are given under “Experimental Procedure.” The numbers are optical density values at 260 mμ and are an arbitrary measure of ribonuclease activity.

<table>
<thead>
<tr>
<th>RNase</th>
<th>Optical density at 260 μμ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>μg x 10^-6</td>
</tr>
<tr>
<td>RNase A</td>
<td>66</td>
</tr>
<tr>
<td>RNase E</td>
<td>128</td>
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<tr>
<td>5</td>
<td>218</td>
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<td>10</td>
<td>340</td>
</tr>
<tr>
<td>25</td>
<td>620</td>
</tr>
</tbody>
</table>

FIG. 7. The regeneration of ribonuclease activity after the separation of RNase E into a trichloroacetic acid-soluble component (E-peptide) and a trichloroacetic acid-insoluble component (E-protein). The figure shows the per cent of the activity of an equivalent amount of RNase E, when varying amounts of E-peptide are added to 5 μl of a solution of E-protein at the same molar concentration. The assays were performed at pH 5 with ribonucleic acid as substrate. E-peptide and E-protein were prepared according to the procedure of Richards for the preparation of S-peptide and S-protein (25). TCA, trichloroacetic acid.
TABLE II
Amino acid composition of ribonuclease and various degradation products.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>E-Peptide</th>
<th>S-Peptide</th>
<th>E-Protein</th>
<th>S-Protein</th>
<th>RNase E</th>
<th>RNase B</th>
<th>RNase A (literature)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>residues/molecule</td>
<td>residues/molecule</td>
<td>residues/molecule</td>
<td>residues/molecule</td>
<td>residues/molecule</td>
<td>residues/molecule</td>
<td>residues/molecule</td>
</tr>
<tr>
<td>Lysine</td>
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<td>2.0</td>
<td>8.2</td>
<td>8.1</td>
<td>10.8</td>
<td>10.5</td>
<td>10.5</td>
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<td>Histidine</td>
<td>0.9</td>
<td>1.0</td>
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<td>3.0</td>
<td>3.9</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.9</td>
<td>1.0</td>
<td>3.0</td>
<td>3.0</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Aspartic acid</td>
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<td>1.0</td>
<td>13.7</td>
<td>14.1</td>
<td>15.2</td>
<td>15.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Threonine</td>
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<td>1.9</td>
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<td>7.7</td>
<td>9.8</td>
<td>9.3</td>
<td>9.3</td>
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<tr>
<td>Serine</td>
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<td>10.3</td>
<td>10.1</td>
<td>13.1</td>
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<td>Glutamic acid</td>
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<td>8.8</td>
<td>12.2</td>
<td>11.5</td>
<td>11.5</td>
</tr>
<tr>
<td>Proline</td>
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<td>4.2</td>
<td>3.7</td>
<td>4.3</td>
<td>4.3</td>
<td>4.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Glycine</td>
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<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.5</td>
<td>5.0</td>
<td>7.0</td>
<td>7.2</td>
<td>10.6</td>
<td>12.0</td>
<td>12.0</td>
</tr>
<tr>
<td>Half-cystine</td>
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<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Valine</td>
<td>8.6</td>
<td>9.2</td>
<td>8.6</td>
<td>9.3</td>
<td>9.3</td>
<td>9.3</td>
<td>9.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.0</td>
<td>1.0</td>
<td>3.0</td>
<td>4.2</td>
<td>4.0</td>
<td>4.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Leucine</td>
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<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>5.7</td>
<td>5.9</td>
<td>5.6</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.9</td>
<td>1.0</td>
<td>2.0</td>
<td>2.0</td>
<td>3.1</td>
<td>3.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Fig. 8. The ribonuclease activity of varying amounts of material corresponding to Peak I of Fig. 1 which has been incubated either in the presence or in the absence of trypsin as indicated under “Experimental Procedure.”

concluded that RNase E differs from RNase S in that an alanine residue at position 20, or the dipeptide alanylalanine at positions 19 and 20, has been cleaved out of the molecule by elastase. Paper chromatographic analysis of aliquots of a digestion mixture taken as a function of time shows that free alanine is produced at least as quickly as any other ninhydrin-positive spot. On the other hand, alanylalanine is never found. Therefore, it is concluded that RNase E is formed with the concurrent release of alanine at position 20 alone. The rather low values for alanine in the analyses of E-peptide and RNase E are considered to reflect either a partial degradation of RNase E subsequent to its formation or experimental error.

On the basis of the preceding evidence, it is concluded that RNase E is produced as the result of the breakage of two peptide bonds, rather than one, in the ribonuclease molecule. If this be correct, it may be predicted that a precursor of RNase E, in which only one peptide bond has been broken, must be formed. This precursor should be characterized by having an NH₂-terminal alanine in addition to lysine, and should be detectable if the rate of its conversion to RNase E is not extremely high.

Since RNase E does not show any NH₂-terminal alanine on end group analysis, it was felt that the precursor of RNase E might be hidden by the RNase A peak on the Amberlite XE-64 column elution patterns. Accordingly, a sample of material emerging at the position of RNase A after elastase digestion (Peak I of Fig. 1) was examined and was found to contain NH₂-terminal alanine in addition to lysine. Thus, a precursor of RNase E does indeed accumulate in detectable amounts, since the starting material has only a single NH₂-terminal group; namely, lysine.

A further indication that Peak I of Fig. 1 is a mixture of RNase A and a modified ribonuclease was obtained on examination of the susceptibility of this material to tryptic hydrolysis. It will be recalled that RNase A is unaffected by trypsin treatment whereas RNase E loses all of its activity when incubated for 1 hour at 37°C with trypsin (Table I). When a sample of material from an elastase digest which moves on Amberlite XE-64 as RNase A was treated with trypsin under the same conditions an appreciable portion of its activity was lost (Fig. 8). It is therefore clear that in the course of the elastase-catalyzed hydrolysis of ribonuclease two protein intermediates accumulate. Both species exhibit ribonuclease activity and both are characterized by an increased sensitivity to trypsin digestion. They differ in their end groups and in their chromatographic mobility and are considered to be the products of one and of two bond breaking steps, respectively.

**DISCUSSION**

RNase E is produced from RNase A as the result of the cleavage of two closely spaced peptide bonds. It is possible to detect a lag in the rate of appearance of RNase E which indicates that a precursor of different chromatographic mobility may exist. Such a precursor has been found to be present in appreciable amount and is shown to contain an alanine end group. Therefore, in the elastase-catalyzed hydrolysis of RNase A, the first bond broken is considered to generate an alanine end group. This occurs as the result of proteolytic attack at the Ala-Ala bond (positions 19 and 20) as shown in Fig. 9. The product of this first step has the chromatographic characteristics of RNase A, but is trypsin-sensitive and has a new alanine end group in its end group analysis, it was felt that the precursor of RNase E might be hidden by the RNase A peak on the Amberlite XE-64 column elution patterns. Accordingly, a sample of material emerging at the position of RNase A after elastase digestion (Peak I of Fig. 1) was examined and was found to contain NH₂-terminal alanine in addition to lysine, and will be called RNase I. This material has properties in between RNase A and RNase E, as might be expected of an intermediate species. The conversion of RNase I to RNase E takes place on hydrolysis of the Ala-Ser bond at positions 19 and 20 along with the concomitant release of free alanine.²

As indicated in Fig. 9, the subtilisin-catalyzed conversion of RNase A to RNase E is accompanied by the appearance of a species which migrates faster than RNase A but slower than RNase E and which has the chromatographic characteristics of RNase I. This species is therefore considered to be the precursor of RNase E. The precursor is formed by the cleavage of two peptide bonds, rather than one, in the ribonuclease molecule. If this be correct, it may be predicted that a precursor of RNase E, in which only one peptide bond has been broken, must be formed.

² It is perhaps surprising that elastase should catalyze the hydrolysis of an external peptide bond. Contamination of elastase with an exopeptidase is a possibility which can be ruled out, since neither carboxypeptidase nor aminopeptidase is inhibited by diisopropyl fluorophosphate, and elastase, after diisopropyl fluorophosphate treatment, is completely inert towards a mixture of peptides derived from ribonuclease by prior elastase treatment. Pepsin has also been shown to act as an exopeptidase with the proper substrate (27) as has chymotrypsin (28).
RNase A to RNase S is also attributable to the hydrolysis of the Ala (20)-Ser (21) peptide bond. It is remarkable that the two proteolytic enzymes, subtilisin and elastase, degrade ribonuclease in such a very similar manner. The explanation of this coincidence is presumably to be found in the structure of ribonuclease. A long sequence of amino acids composed only of alanine, serine, and threonine residues, as is the region of the ribonuclease molecule between positions 16 and 25, is rather unusual. By analogy with the myoglobin structure (29), it may be suggested that this cluster of amino acids will be on the surface of the ribonuclease molecule in full contact with the solvent. The fact that both subtilisin and elastase preferentially attack the ribonuclease molecule in this highly hydrophilic region is in accord with such speculations. Fig. 9 has been drawn so as to suggest, in diagrammatic fashion, a possible structure for this region of the molecule. In the figure the hydroxyamino acids in the sequence in question are drawn as part of an extensively hydrogen-bonded structure of the beta type (30). Because the two alanine residues lack a side chain hydroxyl group they are assumed not to take part directly in these interactions, but they are used to turn the corner in this hairpin type structure. The peptide bonds at the Ala-Ala sequence would therefore be easily available to proteolytic attack, although the other peptide bonds in this sequence would not be susceptible to proteolysis due to the marked lowering of thermodynamic activity which results from their participation in this highly ordered structure.

Model building has shown that Residues 14 to 18 and 21 to 24 can be arranged in an antiparallel beta structure with hydrogen bonding between the amide nitrogen of Residues 14 to 18 and the backbone carbonyl oxygen of Residues 21 to 24. This structure is stabilized by four additional hydrogen bonds which form between the side chain hydroxyl groups of Residue pairs 14 and 16, 15 and 17, 21 and 23, and 22 and 24, respectively. In the last mentioned pair, the side chain carbonyl oxygen of asparagine (Residue 24) takes the place of an hydroxyl group with no distortion of the over-all structure. The three peptide bonds at the Ala-Ala sequence are completely unbonded in this model and leave considerable conformational mobility to these 2 amino acid residues.

Aside from the fact that this model accounts, in a satisfactory manner, for the special nature of the peptide bonds surrounding the Ala-Ala sequence, it is also attractive in that it gives a functional meaning to the extensive cluster of hydroxyamino acid residues in this portion of the molecule. There is ample chemical evidence which indicates that hydroxyamino acids readily interact with one another even in aqueous solvents. For example, poly-L-serine has been found by Bohak and Katrabai to be highly insoluble not only in water but in almost all solvents (31), while Fruton has reported that even the dipeptide L-seryl-L-serine is only sparingly soluble in water (32). Furthermore, hydroxyamino acids seem to exhibit disruptive effects on helices (29), and in synthetic polypeptides lend themselves readily to the formation of beta structures but not of helices (31, 32).

The model imposes some restrictions on the conformational possibilities of ribonuclease. For example, it is found that tyrosine at position 25 and aspartic acid at position 14 cannot participate in a phenol-carboxylate hydrogen bond without destroying the rest of the structure. The two amino acids are, however, brought into close enough juxtaposition to form a backbone hydrogen bond. This result is of interest since Cha and Scheraga have shown that tyrosine at position 25 is one of the three buried tyrosines of ribonuclease (34), and since it is commonly held that these buried residues may be participating in a phenol-carboxylate interaction (35, 36). The model building studies rule out, on steric grounds, one such possible interaction. However, more direct evidence for the existence of the beta structure in ribonuclease is clearly necessary before it can be considered as anything more than a working hypothesis. It comes as no surprise that RNase E, which is missing 1 or 2 amino acid residues from the COOH-terminal region of the peptide portion, has all of the properties of RNase S (which is not missing any of the amino acids of ribonuclease). Hofmann et al. have shown that a peptide containing only the first 13 amino acids of S-peptide can substitute with reasonably high efficiency for the natural peptide in activating S-protein (37). Furthermore, Richards (25) and Potts et al. (38) have shown that a number of amino acids may be removed from the COOH-terminal end of S-peptide without loss in activity. Thus, the carboxyl-terminal region of S-peptide is clearly unessential for the formation of an active ribonuclease molecule. It does, however, seem to play some role in maintaining the stability of the molecule.

It is curious that RNase E migrates chromatographically with the mobility of RNase S, although RNase I moves at the position of RNase A. Since RNases E, S, and I all are products of internal proteolysis, they each possess an extra set of terminal amino and carboxyl groups. It is clear, therefore, that this fact alone cannot account for the lowered mobility of RNase E and RNase S. In support of this deduction is the fact that at pH
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6.5 there should be no appreciable change in the net charge of the molecules due to the new $\alpha$-amino and carboxyl groups since both will be highly ionized. Nor can the removal of alanine account for the lowered mobility of RNase E since RNase S, which is not missing this amino acid, also moves slowly compared to RNase A. It is more likely that the altered mobility of RNase E, as well as that of RNase S, is a manifestation of a conformational transition which accompanies the rupture of the Ala–Ser bond at positions 20 and 21. Spectral evidence for such a conformational change has been obtained by Richards and Logue with RNase S (39). Furthermore, Singer and Richards have shown that RNase S is antigenically different from RNase A (40).

It may be suggested that RNase I has retained, in large measure, the conformation of RNase A since it has an unchanged chromatographic mobility. However, the fact that RNase I is sensitive to trypsin at 37°C indicates that while its conformation is very similar to that of RNase A during chromatography at low temperatures, it becomes more disordered at higher temperatures. A direct comparison of the conformational stability of RNases A, I, S, and E would be highly instructive.

SUMMARY

Bovine pancreatic ribonuclease is readily and extensively hydrolyzed by porcine pancreatic elastase. The reaction involves an initial lag phase and proceeds ultimately to break more than 20 peptide bonds in the ribonuclease molecule. The lag is present when chromatographically purified elastase is used and is seen regardless of whether the reaction is followed titrimetrically or by ninhydrin analysis of amino group liberation. Chromatography of a sample of elastase-treated ribonuclease demonstrates the presence of a new, enzymatically active, species of ribonuclease which is retarded on Amberlite XE-64 relative to ribonuclease A and which has been called ribonuclease E.

RNase E is shown to arise as the result of the elastase-catalyzed hydrolysis of two adjacent peptide bonds near the amino terminus of ribonuclease. The molecule of RNase E is composed of two polypeptide chains which are held together by noncovalent interactions. The modified enzyme retains full activity; it may be resolved into its inactive components and then reconstituted on mixing these components in equimolar amounts. Amino acid analysis shows that RNase E differs from RNase S (subtilisin modified ribonuclease) only in that an alanine residue has been eliminated from the carboxyl terminus of the peptide moiety. A model is proposed for the structure of a portion of the ribonuclease molecule which helps account for the nature of the intermediates formed during the elastase- and subtilisin-catalyzed hydrolysis.

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