Inhibition of Ribonuclease by Copolymers of Glutamic Acid and Aromatic Amino Acids

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(Received for publication, July 26, 1961)

The inhibitory influence of various acidic macromolecules on the enzymatic activity of bovine pancreatic ribonuclease has been studied extensively in recent years. Heparin (1-5) as well as other sulfated polysaccharides (5-7), synthetic polysaccharide macromolecules (8, 9), polyphlorhizin phosphate (4, 10), polyxenyl phosphate (11), and acidic polymers related to humic acids (12) are among the inhibitors that have been investigated. Vandesnieste (4) observed that poly-l-aspartic acid, like other acidic polymers, inhibited the activity of ribonuclease (4). The addition of poly-L-ornithine reversed the inhibition. In all of the above cases, the interaction of the inhibitors with ribonuclease was of an electrostatic character, in which the polyanionic macromolecules reacted with an oppositely charged protein.

It is the purpose of the present investigation to elucidate in what way the presence of amino acids such as tyrosine or phenylalanine in a copolymer with an acidic amino acid would influence the inhibitory capacity toward RNase. The experiments described below indicate that the copolymer of tyrosine and glutamic acid studied is a much more efficient inhibitor of RNase than polyaspartic acid, and that in this case the attachment to the enzyme occurs not only through electrostatic interactions but also through short-range urea-labile bonds.

EXPERIMENTAL PROCEDURE

Materials—Copolymers of L-tyrosine and L-glutamic acid with a residue molar ratio of 1:1.1 (number average degree of polymerization, n 31), of L-tyrosine and L-glutamic acid with a residue molar ratio of 1:0.9 (n 88), of L-phenylalanine and L-glutamic acid with a residue molar ratio of 1:0.87 (n 143), and of L-tyrosine, L-leucine, and L-glutamic acid in a residue molar ratio of 1:1:1 (n 39) have been described previously (13). Poly-L-tyrosyl gelatin, containing 16% tyrosine, was prepared similarly to other polytyrosyl gelatins (14). Poly-l-aspartic acid (n 200) and poly-l-lysine hydrobromide (n 60) were obtained from the Department of Biophysics, The Weizmann Institute of Science.

Bovine pancreatic RNase (“chromatographic grade,” Lot R-60B204) was obtained from Sigma Chemical Company. Ribonuclease acid was prepared from yeast by the method of Crestfield, Smith, and Allen (15). Barium uridine-2',3'-cyclic phosphate was purchased from Schwarz BioResearch, Inc.

Ribonuclease Assays—When ribonuclease acid was used as a substrate, RNase was assayed either by the method of Anfinsen et al. (16) or by a slight modification of the spectrophotometric method of Kunitz (17). In the first case 4 μg of RNase were used in each assay, the weight ratio of substrate to enzyme being constant at 1000:1. The inhibitor was added as a 0.01 or 0.1% solution to RNase before the ribonuclease acid, except where otherwise mentioned.

The Kunitz assays were performed with 0.1% ribonuclease acid in 0.1 M sodium acetate buffer, pH 5. The weight ratio of substrate to enzyme was either 1000:1 or 300:1. The decrease in optical density at 300 μm during the digestion was followed, starting 25 seconds after the mixing of the reactants, in a Cary model 14 M recording spectrophotometer. Relative specific activities were calculated from the slopes which were nearly linear in the first few minutes of the reaction.

For assay with barium uridine-2',3'-cyclic phosphate as substrate, 75 μg of RNase in 1.5 ml of 0.1 M sodium acetate buffer, pH 5, (with or without added inhibitor within the same volume) were mixed with 1.5 ml of the substrate solution (0.4 mg per ml, in the same buffer), in a 3-ml quartz cuvette, and the optical density increase was followed in the Cary spectrophotometer at 280 μm. Specific activities were again calculated from the initial slopes.

RESULTS

Anfinsen Assay—A typical experiment, in which the Tyr-Glu copolymer (1:1.1), the Phe-Glu copolymer, and polyaspartic acid were used as inhibitors of RNase, is shown in Fig. 1. The copolymer with tyrosine is a much more efficient inhibitor than polyaspartic acid. The copolymer with phenylalanine is also a significantly better inhibitor than polyaspartic acid, but is much less efficient than the copolymer with tyrosine.

A copolymer of tyrosine, leucine, and glutamic acid (1:1:1) caused 50% inhibition at an inhibitor to enzyme (weight per weight) ratio of 20, whereas a copolymer containing tyrosine and glutamic acid in a 1:9 residue molar ratio was only slightly more effective than polyaspartic acid; i.e., at a ratio of 50 the activity was still 65% of that of the native RNase in the absence of any inhibitor. Polytyrosyl gelatin was assayed as an example of a water-soluble neutral macromolecule containing tyrosine peptides. It was completely ineffective.

The Tyr-Glu copolymer (1:1.1) is also an efficient inhibitor when added to a mixture containing both ribonuclease acid and RNase. In the Anfinsen assay the enzymatic reaction is stopped after 25 minutes by addition of uranyl acetate. Fig. 2 shows the results of an experiment in which 100 μg of the copolymer (25 times the amount of the enzyme) were added to each assay at various times. It is apparent that the inhibition was complete...
and instantaneous, as the curve is similar to that obtained when uranyl acetate is used to stop the reaction at various times.

The inhibition of RNase by the Tyr-Glu copolymer may be reversed by means of polylysine. At an inhibitor (I) to enzyme (E) ratio of 5, the activity is 44% in the absence of polylysine, but 82% in the presence of a 5-fold excess of polylysine hydrobromide over the inhibitor. At a 10-fold excess the activity was 94%, i.e. it was practically completely restored.

Kunitz Assay—The inhibition of RNase by the Tyr-Glu copolymer (1:1.1), assayed according to Kunitz, is shown in the bottom curve of Fig. 3. The results were the same, whether the weight ratio of substrate to enzyme was 300 or 1000.

At a weight ratio of I:E equal to 4, the inhibition is almost complete when followed by the Kunitz assay, but there is still 50% activity, according to the assay with uranyl acetate precipitation. This difference in the behavior of the inhibitor emphasizes that the two assay methods measure different aspects of the over-all reaction.

When the digestion of ribonucleic acid by RNase was followed spectrophotometrically, the addition of the inhibitor at I:E = 10 was enough to stop the reaction completely, whereas at I:E = 4 the activity was reduced to 74% of that in the absence of the copolymer.

Uridine-2',3'-cyclic Phosphate as Substrate—The Tyr-Glu copolymer (1:1.1) is a very good inhibitor of RNase, when the activity is measured with the use of the barium salt of uridine-2',3'-cyclic phosphate as substrate (Fig. 4). In this case an amount of the inhibitor equal to that of the enzyme in the assay sufficed to cause more than 50% inhibition, whereas a 4-fold excess abolished the activity completely.

Inhibition in 8 M Urea—Since RNase is active in 8 M aqueous urea, inhibition experiments could be performed in this solvent by using the Kunitz assay. There is no significant difference between the effectiveness of polyaspartic acid as an inhibitor of RNase in a sodium acetate buffer of pH 5 in the presence or absence of urea (Fig. 3, upper curve). On the other hand, the Tyr-Glu copolymer (1:1.1) lost most of its effectiveness in 8 M urea, and its behavior then resembles, closely, that of polyaspartic acid. The Phe-Glu copolymer, similarly, lost most of its inhibitory capacity when the reaction mixture was exposed to 8 M urea. Thus the activity of ribonuclease, by the Kunitz assay, in the presence of Phe-Glu copolymer, at inhibitor to enzyme ratios of 20 and 50, was 0 in 0.05 M sodium acetate buffer, pH 5, and 69% and 50%, respectively, in the same buffer containing 8 M urea.

pH Dependence of Inhibition—All of the experiments described above were carried out at pH 5. In agreement with reports on other polyanionic inhibitors (1, 2, 12) the capacity of the Tyr-Glu copolymer (1:1.1) to inhibit RNase diminishes dramatically as the pH is raised. At pH 5.0, and a ratio of inhibitor to enzyme of 1, inhibition of 47% was observed. The decrease in inactivation with increasing pH is shown in Fig. 5 for weight ratios of I:E of 10 and 50. Activities were estimated by the Kunitz assay in 0.1 M Tris-malonate buffers.

**Discussion**

Acidic macromolecules inhibit RNase through electrostatic competition with the substrate for the enzyme. The experiments reported in this paper show that the negative charges must be present in the molecule for the inhibition to occur at all. The presence of phenylalanine and, especially, tyrosine in the anionic polymer increases, dramatically, its inhibitory capacity. Table I summarizes the experimental data. The negative charge appears to be necessary to allow the polymeric inhibitor to approach the enzyme and bind to it through long range electrostatic forces; tyrosine or phenylalanine may then form additional short range uren-labile bonds. The nature of these bonds (hydrophobic bonds, hydrogen bonds in the case of tyrosine, etc.) is not apparent from this study. Nor is it possible to specify the groupings in the RNase molecule which are involved. One plausible speculation would involve the interaction with tyrosine residues in the enzyme. Some tyrosines are known to participate either in the active center of RNase or in bonds concerned with stabilizing the molecule in such a configuration that the catalytic center may be active (18–25).

The amino acid residues in the copolymers used in this study have a relatively random distribution along the chain (26). The observation that a copolymer containing, on the average, only 1
tiryosyl residue in 10 is a poor inhibitor suggests that in the inhibition by means of the Tyr–Glu copolymer (1:1.1) tyrosine peptides play a role rather than unique tyrosine residues. A similar situation has been described in the studies on the inhibition by means of tyrosine- or phenylalanine-containing copolymers of the immune reaction between polytryosyl or polyphenylalanyl gelatin and their respective antibodies (13).

The synthetic inhibitors described are composed of L-amino acids linked through peptide bonds. The Tyr–Glu copolymer (1:1.1), at pH 5.0, one of the most powerful inhibitors of RNase. It is interesting that some of the good RNase inhibitors such as polyphloretin phosphate (4, 10), polyxenyl phosphate (11), and some acidic polymers related to humic acids (12) contain in their molecules aromatic rings as well as negative charges. The Anfinsen assay measures the amount of oligonucleotides small enough not to be precipitated by uranyl acetate under the conditions used. The Kunitz assay measures a spectral shift. Levels of copolymers which caused complete inhibition when the reaction was followed by the Kunitz method decreased RNase activity only partially when checked by the uranyl acetate method. It seems thus that the Kunitz assay measures the spectral changes occurring upon cleaving of small oligonucleotides into even smaller units, and that this cleaving is inhibited completely at an inhibitor level which causes only a decrease of the cleaving of ribonucleic acid into oligonucleotides.

The increase in the inhibitory efficiency when uridine-2',3'-cyclic phosphate is used as a substrate, rather than ribonucleic acid, is most likely due to the more successful competition of a polymeric acid against the low molecular weight substrate, than against the high molecular weight macromonomion. The decrease in inhibitory capacity as the pH of the reaction is raised from pH 5.0 to pH 7.5 has been reported previously (1, 2, 11, 12). Litt (27), studying the kinetics of RNase action on cytidine-2',3'-cyclic phosphate, has reported recently that the products inhibit the reaction strongly at pH 5.0 and 6.0, but

![Figure 3](image3.png)

**Fig. 3.** Kunitz assay of RNase in presence of: ○, Tyr-Glu copolymer (1:1.1); △, Tyr-Glu copolymer (1:1.1) in 8 M urea; ▲, poly-L-aspartic acid; Δ, poly-L-aspartic acid in 8 M urea.

![Figure 4](image4.png)

**Fig. 4.** The assay of RNase using the barium salt of uridine-2',3'-cyclic phosphate, in the presence of increasing amounts of Tyr-Glu copolymer (1:1.1).

![Figure 5](image5.png)

**Fig. 5.** The extent of inhibition of RNase by Tyr-Glu copolymer (1:1.1) as a function of the pH; ○, at an inhibitor (I) to enzyme (E) ratio of 10; O, at an I:E of 50.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Assay</th>
<th>1:E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copolymer Tyr-Glu (1:1.1)</td>
<td>Anfinsen</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Kunitz</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Synthetic substrate</td>
<td>0.9</td>
</tr>
<tr>
<td>Poly-L-aspartic acid</td>
<td>Anfinsen</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>Kunitz</td>
<td>35</td>
</tr>
<tr>
<td>Copolymer Tyr-Leu-Glu (1:1.1)</td>
<td>Anfinsen</td>
<td>20</td>
</tr>
<tr>
<td>Copolymer Phe-Glu (1:0.87)</td>
<td>Anfinsen</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Kunitz</td>
<td>10</td>
</tr>
</tbody>
</table>
not at pH 7.0 and 8.0. Because this effect has been observed with heparin, it seems improbable that the state of ionization of the inhibitor is responsible for the pH dependence. The ribonucleic acid is also fully ionized in the pH range investigated, and even though Zillner and Fellig (1) have suggested that the pH of biological activity will not often increase their efficiency.

A more plausible alternative would involve some dissociable groups on the enzyme, such as carboxyl or imidazole groups with the secondary phosphoryl groups of pyrimidine nucleotides. The change in the enzyme ionization would then have to result in a decreased binding of the inhibitor as compared to the substrate.

It is interesting, even though possibly only coincidental, that the pH dependence of the recovery of active RNase upon reoxidation of the fully reduced molecule follows approximately the same curve as that in Fig. 5.1 The Tyr–Glu copolymer (1:1.1) is also a very efficient inhibitor of the recovery of the active enzyme during the reoxidation. This reaction is carried out at a slightly alkaline pH at which the copolymer has no inhibitory capacity toward RNase.

Should copolymers of glutamic acid with aromatic amino acids be efficient inhibitors of RNases from sources other than the bovine pancreas, they might possibly find a limited use in the isolation of ribonucleic acids.

Finally, the speculation may be raised on whether the inclusion of aromatic amino acids within the synthetic polyelectrolyte macromolecules, that have been shown to possess various types of biological activity (28), will not often increase their efficiency.

SUMMARY

Copolymers containing approximately equal molar ratios of glutamic acid and tyrosine or phenylalanine are much more effective inhibitors of ribonuclease than polyaspartic acid or a copolymer of glutamic acid and tyrosine in a molar ratio of 9:1. These results in inhibitor efficiency seem to be due to an interaction between the aromatic amino acids and groupings in the ribonucleic molecule, over and above the long-range electrostatic interaction between the inhibitor and the enzyme which is responsible for the inhibitory capacity of polyamions in general. The specific short-range bonds are disrupted by 8 M urea, in which the copolymers studied are not better inhibitors than other acidic macromolecules lacking the aromatic side chains.

In agreement with other reports, the inhibition of ribonuclease by the copolymers studied decreases strongly as the pH is raised. The digestion of ribonucleic acid by ribonuclease may be stopped completely, at pH 5.0, by relatively small amounts of the inhibitory copolymer.

REFERENCES

Inhibition of Ribonuclease by Copolymers of Glutamic Acid and Aromatic Amino Acids
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