Stabilization of Pancreatic Ribonuclease against Urea Denaturation by Anion Binding

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Partial renaturation of urea-denatured bovine pancreatic ribonuclease by polyanions such as phosphate and polyphosphates, as well as by nucleotides, has been observed by Sela, Anfinsen, and Harrington (1). The finding that the velocities of denaturation and renaturation of ribonuclease may be separately and easily measured (2) prompted us to reinvestigate by kinetic methods the manner by which anions impart stability to the native form of the enzyme in the presence of denaturing concentrations of urea. As expected, a number of different anions were found to inhibit the denaturation rate. In considering an analogous stabilization by calcium or strontium ions of the native structure of chymotrypsin against urea denaturation, Chervenka (3) employed a simple expression given by Johnson et al. (4) by which not only the dissociation constant of the protein-inhibitor complex but also the number of inhibitor molecules bound per molecule of protein can be determined readily. This paper presents evidence, based upon similar kinetic studies of the inhibition of ribonuclease denaturation, measurements of competitive inhibition of ribonuclease activity, and dialysis equilibrium experiments, that stabilization of the native form of ribonuclease involves binding of a single anion molecule to the catalytic site of the enzyme. From a comparison of the effect of pH upon the binding of different anions, certain ionization properties of the cationic groups at the catalytic site of ribonuclease were shown.

EXPERIMENTAL PROCEDURE

Materials

The preparation and standardization of pancreatic RNase A, dialyzed desalted commercial RNase buffers, and urea solutions have been described previously (2). RNase A was used throughout except for the determination of the pH dependence of the inhibition of denaturation by phosphate and sulfate ions (Fig. 4), in which case dialyzed desalted commercial RNase was used.

Barium uridine 2',3'-cyclic phosphate (U-cyclic-p, Schwarz Laboratories) containing 10% of 2'- and 3'-UMP was purified by column chromatography according to Brown, Dekker, and Todd (5). Fifty milligrams were placed on a Dowex 1-X10 (200 to 400 mesh) resin column (0.9 cm × 13 cm) and eluted with a buffer containing 0.01 M formic acid and 0.05 M ammonium formate (pH 4.2) at a flow rate of 32 ml per hour while collecting 11 ml per tube. The U-cyclic-p appeared in tubes 28 to 46 while 2'- and 3'-UMP appeared in tubes 62 to 84. The tubes containing U-cyclic-p were frozen and stored for use as stock solutions after standardization according to its molar extinction of 9180 at 260 mμ (6). Isolation of 2'-CMP was carried out as previously described (7).

For dialysis equilibrium experiments, it was necessary to determine the equilibrium concentration of 2'-CMP by a procedure that did not involve spectrophotometric measurements, because small but significant amounts of soluble ultraviolet-absorbing materials leached out of the dialysis tubing. For this purpose, 2'-CMP-2-C14 was used. It was isolated from a mixture containing 9 mg (1.18 με per mg) of mixed 2'- and 3'-isomers of cytidylic acid-2-C14 (Schwarz Bio-Research, Inc.) and 90 mg of unlabeled 2'- and 3'-isomers of CMP (California Corporation for Biochemical Research) by elution from Dowex 1 (8).

Methods

Denaturation and Renaturation Kinetics—The velocity of denaturation or renaturation was measured by the change in absorbance at 287.5 mμ and by the change in optical rotation at 365.5 mμ as previously described (2). In a few studies, the change in absorbancy at 235 mμ that accompanies the reversible unfolding of RNase (9) was also measured. In studies of the rate of urea denaturation of RNase in the presence of 2'-cytidylic acid, the high extinction of the nucleotide prevented the use of spectrophotometric measurements. Optical rotation determinations were employed because at the highest concentration (1.2 × 10-4 M) of 2'-CMP used, its contribution to the rotation was only 2% of that of the RNase. The RNase concentration employed was 3 to 4 × 10-4 M for all spectrophotometric measurements and 1.3 × 10-4 M for optical rotation measurements, except where otherwise indicated.

RNase Activity—The initial velocity of hydrolysis of U-cyclic-p to 3'-UMP was measured by the increase in absorbancy at 290 mμ as described by Richards (10). An increase of about 0.03 absorbancy unit in 100 seconds was observed when 0.7 to 5 × 10-4 M U-cyclic-p was hydrolyzed by 1.5 × 10-4 M RNase at pH 7.3. Under the conditions employed here, no spectral interaction between U-cyclic-p and RNase of the type described previously (7) was observed. Only the initial rate...
was measured so that no significant amounts of inhibitory 3'-UMP would be formed or would interact with RNase to cause a spectral change. All denaturation and enzymatic reactions were carried out at 30 ± 0.1° except where otherwise indicated.

**Dialysis Equilibrium**—Dialysis equilibrium experiments were performed with bags of 1/2-inch dialysis tubing (Fisher Scientific Company) 1 inch long. The knotted end was inserted into 2-dram vials with snap-on caps (T. C. Wheaton Company, Millville, New Jersey), and the open end was pulled down around the rim. The vial contained 2 ml of a solution containing 0.5 to 5.0 × 10⁻⁴ M 2'-CMP-2-C³⁴ in acetate, imidazole, or Tris buffers of 0.1 ionic strength. One milliliter of a solution containing 1.7 × 10⁻⁴ M RNase A in the same concentration of 2'-CMP-2-C³⁴ and buffer was placed inside the dialysis bag. Each concentration of 2'-CMP was studied in quadruplicate.

To correct for the changes in 2'-CMP-2-C³⁴ concentration due to dilution by the moisture of the casing as well as by adsorption, vials were also assembled in which RNase had been deleted from the solution inside the bag. The vials were closed off and the dialysis tubing was secured firmly in place by tightly fitting polyethylene caps. The vials were placed on their sides in a device that rotated them at a rate of 2 r.p.m. After 6 hours at 27°, when equilibrium had been achieved but before any significant amount (less than 0.1%) of RNase had escaped across the membrane (as tested by the method of Lowry et al. (11) for protein), quadruplicate aliquots were evaporated to dryness in counting vials, the residue was dissolved in 0.5 ml of 1 M Hyamine (Packard Instrument Company, LaGrange, Illinois) and 10 ml of the scintillation counting fluid described by Chen (12) were added. Each vial was counted in a Packard Tri-Carb liquid scintillation spectrometer for 30 minutes. The counting rate of the samples was from 4- to 40-fold greater than the background rate. On addition of internal standard, it was found that corrections for quenching were unnecessary.

**RESULTS**

As anticipated from the observation by Sela et al. (1) that polyanions stabilize the native forms of RNase, we found that phosphate, sulfate, polyphosphate, and a number of other polyvalent anions inhibited the rate of denaturation of RNase by 8 M urea. The treatment by Johnson et al. (4) was useful in interpreting these data. It is assumed that native RNase but not denatured RNase reversibly binds n moles of an inhibitor (I) to form an RNase-nI complex that denatures very slowly as compared to the protein not bound in a complex in the presence of a denaturing agent. This being so,

\[
\log \frac{v_0 - v_I}{v_I} = n \log (I) - \log K_I
\]

in which \(v_0\) is the velocity of the uninhibited reaction, \(v_I\) the velocity in the presence of inhibitor, \(I\) the inhibitor concentration, and \(K_I\) the dissociation constant of the RNase-inhibitor complex. A plot of \(\log (v_0 - v_I)/v_I\) versus \(\log (I)\) should yield a straight line with a slope equal to the number of moles of inhibitor bound per mole of RNase and at 50% inhibition, \(n \log (I)\) equals \(\log K_I\). In actual practice, the first order rate constants for each reaction instead of the initial velocities were employed because they could be more accurately determined. As expected, the percentage of inhibition by 0.002 M phosphate in 7.5, 8.0, 8.5, and 9.0 M urea at pH 7 and 0.04 ionic strength was the same (34, 29, 33, 33%, respectively).

In Fig. 1 are shown the data obtained with pyrophosphate, 2'-CMP, phosphate, citrate, tartrate, and sulfate. The dissociation constants of various RNase-anion complexes, together with the number of anions bound per protein molecule, are summarized in Table I. The standard error in the determination of the dissociation constants was in each case about 15% as determined by least squares. In most cases, the binding of anions

![Graph](image-url)
to RNase was mole for mole within 10 to 15%. This method cannot be used for more powerful inhibitors of the denaturation with dissociation constants less than half of the molar concentration of the enzyme. The three values showing less than equimolar binding were probably affected by changes in the ionic strength contributed by the inhibitor as is described later.

Because 2'-CMP is a potent competitive inhibitor of RNase activity, and because inhibition of denaturation involves binding of a single molecule of 2'-CMP, it seemed likely that binding not only of 2'-CMP but also the other anions takes place at the catalytic site. To test this idea, the inhibition by phosphate, sulfate, and pyrophosphate of the RNase-catalyzed hydrolysis of U-cyclic-p was studied. In Fig. 2, the reciprocal of the initial velocity of the hydrolysis is plotted as a function of the reciprocal of the substrate concentration according to the Lineweaver-Burk equation

$$\frac{1}{v} = \frac{K_m}{V_{\text{max}}} \left( \frac{1}{1 + \frac{I}{K_I}} \right) \frac{1}{S} + \frac{1}{V_{\text{max}}}$$

These anions, which protect RNase from denaturation, were found to be competitive inhibitors of RNase activity, for in each case, the data fell on straight lines with different slopes but a common intercept with those obtained in the absence of inhibitor. The dissociation constants of the RNase-anion complexes found by competitive inhibition of RNase activity (Table I) agree reasonably well with the values obtained by inhibition of denaturation. Since the ionization of groups on both the enzyme and the inhibitors was probably depressed to varying degrees in concentrated urea solution, exact agreement should not be expected even if the binding phenomena measured are identical.

The inhibition of RNase denaturation by simple anions was reduced at high ionic strengths (Tables I and II), as anticipated from the well known diminution of electrostatic interactions by high concentrations of electrolytes. The curvature of the plot of the inhibition of denaturation by citrate shown in Fig. 1 may have been due to its ionic strength contribution at higher citrate concentrations so that binding was thereby weakened. In the determination of dissociation constants from denaturation inhibition, contributions to the ionic strength from the enzyme and the inhibitors were probably depressed to varying degrees in concentrated urea solution, exact agreement should not be expected even if the binding phenomena measured are identical.

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The greater degree of binding by RNase of 2'-CMP than of 2'CMP is only slightly affected by high ionic strengths, showing that additional nonelectrostatic binding forces must be involved in this case. A comparison of the effect of the various anions as inhibitors of RNase activity or denaturation suggested that their action might be related to the well known Hofmeister or lyotropic series. This is generally given as: citrate > phosphate > tartrate > sulfate > fluoride > acetate > chloride > nitrate > bromide > iodide (15). This seemed to be the case because the rate of urea denaturation of RNase in 1/2 0.01 imidazole buffer at pH 7.3 in the presence of 0.1 M solutions of the sodium salts of various anions was inhibited in the order: sulfate (66%) > fluoride (44%), acetate (42%) > formate (36%), nitrate (35%) > chloride (29%) > bromide (23%) > iodide (16%) (all ±2%), as compared with the rate in the absence of added electrolytes.

These comparative studies of inhibition by anions of RNase activity and of the rate of urea denaturation indicate that one anion is bound per mole of RNase and that the respective binding strengths are comparable. Furthermore, complex formation between RNase and 2'-CMP as measured by spectral changes involves a mole for mole interaction between enzyme and nucleotide (7). This implies that the stabilization of the native structure may be due to binding of a single anion at the catalytic center. Further proof is needed, however, that this...
is not brought about by two separate binding sites on the enzyme which have identical binding affinities. This was established by means of dialysis equilibrium for the RNase-2'-CMP system. As seen in Fig. 3, a maximum of 1 mole of 2'-CMP is bound per mole of RNase at pH 5.6 as the concentration of 2'-CMP is increased. At pH 7.3, the dissociation constant of the RNase-2'-CMP complex as calculated from dialysis equilibrium data (Fig. 4) was 3 × 10^{-4} M, a figure in excellent agreement with the values calculated by competitive inhibition (2.9 M).

**Fig. 3.** Binding of 2'-CMP by RNase as a function of 2'-CMP present, as measured by dialysis equilibrium at pH 5.6, 27°, 0.1 ionic strength. The inner compartment contained 1 ml of 1.85 × 10^{-4} M RNase plus 2'-CMP and buffer; the outer compartment contained 2 ml of 2'-CMP and buffer. The total amount of 2'-CMP added was, therefore, the sum of that in the inner and outer compartments.

**Fig. 4.** Upper, percentage of inhibition of the rate of urea denaturation of RNase as measured spectrophotometrically by 1.85 × 10^{-4} M phosphate and by 1.85 × 10^{-4} M sulfate as a function of pH. An ionic strength of 0.04 was employed, with acetate, imidazole, and Tris buffers for the appropriate pH ranges. Lower, the effect of pH on the binding of 2'-CMP by RNase as measured by dialysis equilibrium, with 1 ml of 1.68 × 10^{-4} M RNase and 1.60 × 10^{-4} M 2'-CMP in acetate, imidazole, or Tris buffer of 0.1 ionic strength in the inner compartment and 2 ml of 2'-CMP-buffer mixture of the same composition in the outer compartment. Also shown is the binding of 2'-CMP by RNase as measured spectrophotometrically, as previously determined by Hummel et al. (7).

**TABLE III**

<table>
<thead>
<tr>
<th>Salt (at 0.1 m)</th>
<th>Rate constant</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>18.0 sec^{-1} × 10^6</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>19.4 sec^{-1} × 10^6</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>14.3 sec^{-1} × 10^6</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>13.2 sec^{-1} × 10^6</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>16.2 sec^{-1} × 10^6</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>17.0 sec^{-1} × 10^6</td>
</tr>
<tr>
<td>Potassium phosphate</td>
<td>15.0 sec^{-1} × 10^6</td>
</tr>
</tbody>
</table>

These are the rate constants of renaturation of RNase as measured spectrophotometrically in solutions containing the above salts in 3 m urea, in addition to 0.1 ionic strength imidazole buffer at pH 7.3.

**TABLE IV**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Rate constant*</th>
</tr>
</thead>
<tbody>
<tr>
<td>255 m°C</td>
<td>5.3 ± 0.2 sec^{-1} × 10^6</td>
</tr>
<tr>
<td>260 m°C</td>
<td>5.3 ± 0.3 sec^{-1} × 10^6</td>
</tr>
<tr>
<td>287.5 m°C</td>
<td>7.0 ± 0.2 sec^{-1} × 10^6</td>
</tr>
</tbody>
</table>

* The rate of renaturation of RNase which had been previously denatured in 5 m urea at 30° for 30 minutes was measured after dilution into 0.5 m urea in 0.1 m acetate, pH 8.0, at 30°. For measurements at 1.2 × 10^{-4} M for those at 235 m°C, it was 2.5 × 10^{-9} M. When 2'-CMP was added, its concentration was equal to that of the RNase.

The pH dependence of the inhibition of denaturation by anions was inspected in an attempt to identify any critical ionizing groups associated with their binding. Phosphate and sulfate both exhibited maxima at pH 5.5 when the percentage of inhibition of the rate of denaturation was plotted against pH, as shown in Fig. 4. The similarity of these two curves from pH 5.5 to pH 9 excludes a decreased ionization of the secondary acid group of phosphate (pK = 7.2 in urea) as being responsible for the change in binding over this pH range, and instead suggests that protonation of groups on the catalytic site rather than on the anionic molecule bring about increased binding as the pH is lowered. For purposes of comparison, the pH dependence of 2'-CMP-RNase interaction as measured spectrophotometrically by Hummel et al. (7) as well as that of the binding of 2'-CMP by RNase by dialysis equilibrium studies are also shown in Fig. 4. The similarity of all of these curves as the pH is varied from 5 to 9 suggests that common cationic groups may be involved.1 The decreased binding by RNase of phosphate or

1 Herries (16) observed a similar maximum at pH 5 of the Michaelis constant for the hydrolysis of C-cyclic-p by RNase, whereas the maximal velocity was greatest at pH 7. This indicates that at pH 5, the Michaelis constant must be essentially a dissociation constant of the enzyme-substrate complex.
2'-CMP, as distinct from sulfate, below pH 5 could be attributed to protonation of the phosphate groups.

In contrast to their inhibitory effect on the velocity of the denaturation of RNase in urea, polyvalent anions (Table III) or 2'-CMP (Table IV) had little or no effect upon the rate of renaturation of urea-denatured RNase which took place when the urea concentration was lowered. This indicates that binding of a virtual substrate cannot serve as a directing influence to hasten the realignment of the unfolded protein but occurs only after the native conformation has been re-established. These findings, together with our observations that the renaturation rate is essentially unaffected by variations in pH and ionic strength (2), emphasize that the forces that serve to realign the polypeptide chain properly are nonelectrostatic in character.

By observing the decrease in the 260-rnp absorbancy which arises from the interaction between 2'-CMP and native RNase during the renaturation of denatured RNase in the presence of 2'-CMP, the rate of the reformation of the nucleotide binding site could be measured. As seen in Table IV, this rate was somewhat faster than the rates of renaturation as measured by the changes in the absorbancy of the protein at 235 and at 287.5 m\mu, either in the presence or the absence of the nucleotide. Glaser and Smith (9) have interpreted the altered absorbancy at 235 m\mu which accompanies protein denaturation as reflecting an altered conformation of the polypeptide backbone. If their view is correct, our data would suggest that realignment of the amino acid residues about the nucleotide binding site proceeds somewhat more rapidly than the refolding of the polypeptide backbone or of the anomalous tyrosine residues in other parts of the molecule.

DISCUSSION

Multiple binding of anions to some proteins is well known (17) and often can either stabilize or weaken the native configuration. On the other hand, specific binding of ionic molecules to the catalytic site of enzymes is usually unimolecular and would not necessarily be expected to impart stability against denaturation. In several cases, notably chymotrypsinogen (3), prostatic phosphatase (18), and glutamic dehydrogenase (19), good evidence for such a simple stabilization has been obtained. If this stabilization of RNase were due to simple counterion shielding of mutually repulsive cationic groups that ordinarily promote the uncoiling of the chain, the inhibitory effects of various anions should not be greatly different.

The stabilizing effects of the various polyvalent anions thus may be ascribed to complex formation involving specific groups at the active center of the enzyme. The observations that the binding is greatest at pH 5, that it is depressed at higher ionic strengths, and that the affinity of the anions follows the Hofmeister electronegativity series signify that it is chiefly electrostatic in character.

The somewhat different actions of RNase upon RNA and upon C-cyclic-p are each competitively inhibited by 2'-CMP, although to different degrees (13). The observation that one and only one molecule of 2'-CMP is bound to RNase effectively disposes of the possibility that RNase might have distinctly separate catalytic sites for these different substrates. The much larger apparent dissociation constant for the RNase-2'-CMP complex, which is kinetically measured in the presence of RNA, as contrasted to the dissociation constants which are obtained in the absence or presence of simple cyclic substrates, probably reflects some supplemental affinity of RNase for RNA by other noncatalytic binding sites in addition to that in the vicinity of the active center.

The binding of RNase of polyvalent anions is very similar to the binding of 2'-CMP. Both bring about competitive inhibition of RNase activity, both are maximal at pH 5, and both protect the enzyme against denaturation. We have observed (7) that complex formation between RNase and 2'-CMP is blocked by addition of phosphate or by carboxymethylation of the enzyme. Moreover, the reaction of bromoacetate with native RNase to form the inactive carboxymethyl-imidazole derivative is blocked by 2'-CMP (20). Taken together, these facts suggest that at least one of the cationic groups involved must be the histidine residue in the vicinity of the catalytic center. Because of the fact that iodoacetamide, as distinct from iodoacetate, did not alkylate the histidine residue of RNase at pH 5, Stark et al. (21) proposed that there is an additional positive charge near the crucial histidine residue in the native protein. The pH sensitivity of the unique alkylation by iodoacetate indicates that this additional cationic group might be ascribed to another histidine or to some other residue with a pK in the neutral region. In support of this idea, Herries (16) deduced from the pH dependence of the Michaelis constant and maximal velocity of the hydrolysis of C-cyclic-p by RNase that the participating cationic groups at the active site of the free enzyme have pK values of 5.22 and 6.78. Furthermore, a positively charged lysine residue might be located near the catalytic center because dinitrophenylation of Lys 41 results in complete inactivation (22).

Regardless of the nature of the other cationic group involved, it seems reasonable to postulate that the native configuration of RNase is less easily disrupted by denaturing treatment because the polypeptide chain is additionally secured in place by the electrostatic affinity of polyvalent anions to two or more cationic groups which lie on either side of a critical seam in the molecule and which make up part of the active center. If this is an accurate picture of the anion binding site, then it seems that the nucleotide substrates are also bound to RNase in such a way that their phosphate groups straddle this seam, spanning several positive charges.

SUMMARY

The inhibition of the rate of denaturation of pancreatic ribonuclease by various anions in concentrated urea solutions was investigated. Changes in the extinction of the enzyme at 287.5 m\mu or in its optical rotation at 360.5 m\mu were employed as criteria of unfolding. In decreasing order, pyrophosphate, 2'-cytidylic acid, phosphate, citrate, tartrate, and sulfate all inhibited the denaturation rate. In all cases except for citrate, the binding appeared to be unimolecular. The effectiveness of other less inhibitory anions in slowing the denaturation rate was a function of their electronegativity. The inhibition of denaturation by anions was decreased at high ionic strengths and was much greater at pH 5.6 than at pH 7.3. Evidence was obtained that the stabilization of the protein is caused by the binding of a single anion molecule. In contrast to their effect on the denaturation reaction, polyvalent anions or ctdylic acid had little effect upon the rate of renaturation of urea-denatured ribonuclease. The rate of reformation of the nucleotide binding site during renaturation was somewhat more rapid than the rate...
of refolding as measured by absorbancy changes in the protein chromophores.

The dissociation constants of the ribonuclease-anion complexes, as measured by denaturation inhibition studies, agreed well with those measured by competitive inhibition of ribonuclease activity upon uridine 2',3'-cyclic phosphate.

Dialysis equilibrium measurements indicate that a maximum of 1 molecule of 2'-cytidylic acid may be bound by ribonuclease. Likewise, as 2'-cytidylic acid has been shown to be a competitive inhibitor of the action of ribonuclease both upon ribonucleic acid and upon simple cyclic phosphate substrates, this observation indicates that the same binding site is involved in both enzymatic reactions.

The effect of pH on the binding by ribonuclease of phosphate and sulfate, as measured by denaturation inhibition, was similar to its effect on the binding by ribonuclease of 2'-cytidylic acid, as measured by dialysis equilibrium or spectral interaction studies, both being maximal at pH 5. This was taken as evidence that protonation of one of the groups on the binding site of the enzyme rather than of a group on the anion is required for the electrostatic interaction. Since binding of a single anion to the active center of the enzyme appears to be responsible for the stabilization of the native structure of ribonuclease in the presence of high concentrations of urea, it is suggested that the active center may lie across a critical seam in the ribonuclease molecule.

REFERENCES

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