Ligand-induced Conformational Changes in Ribonuclease

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SUMMARY

The rate of formation of ribonuclease-S from ribonuclease A by the action of subtilisin was measured at pH 5.5 in the presence and absence of the strongly bound ribonuclease inhibitor, 2'-cytidylate. Presence of the inhibitor in the active center significantly reduced the rate of formation of ribonuclease-S. Since bond 20-21 (and, to a lesser extent, 21-22), the cleavage of which is responsible for the formation of this derivative, is well removed from the active center region, and thus from the binding site of the inhibitor, steric hindrance by the latter can be excluded as the cause for this reduced rate. Instead, it is concluded that the presence of the inhibitor in the active center caused a conformational change which affected a distant region of the molecule. Amino-terminal analysis confirmed cleavage at the expected major site, bond 20-21.

The presence of the competitive inhibitors, 2'- and 3'-cytidylate, afforded considerable protection against inactivation of ribonuclease A by trypsin and by chymotrypsin, when digested at 60°C. End group analysis confirmed that the bonds split were those expected from previous work of others. The presence of 2'-cytidylate completely prevented the digestion at 60°C. End group analysis confirmed that the bonds split were those expected from previous work of others. The presence of 2'-cytidylate completely prevented the digestion at 60°C. End group analysis confirmed that the bonds split were those expected from previous work of others.

The data presented support the view that the presence of the competitive inhibitor in the active center region alters the proteolytic susceptibility of bonds at multiple sites, situated outside of this region.

That the tertiary structure of globular proteins in aqueous solution is not unique, but undergoes fluctuations, is suggested by many observations and has a sound theoretical foundation.

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drastically impair the enzymatic function, were these charges located in the active center itself; (b) it has been shown that elimination by carboxypeptidase of the sequence serine 16 to alanine 20 from the S-peptide did not prevent reconstitution of full enzymatic activity (7); and (c) addition of the synthetic S-peptide fragment consisting of residues 1 to 13 to S-protein resulted in the restoration of full activity (8). The experiments in the present study were designed to test whether the rate of ribonuclease-S formation, i.e. the rate of cleavage of bond 20-21 by subtilisin, was altered by the presence of the strong competitive inhibitor, 2'-cytidylate, in the active center.

It was felt that additional evidence for the effect of ligand remote from its binding site could be obtained by measurements of the rate of cleavage of several additional bonds of known location in the primary sequence. Ooi, Rupeil, and Scheraga (9), and Rupeil and Scheraga (10) showed that at temperatures above 60° both trypsin and chymotrypsin are able to attack ribonuclease, and they identified the bonds broken under these conditions. A study of the effects of bound nucleotides on the rate of cleavage of these bonds by the two proteases seemed therefore informative. If it could be shown that the presence of a single molecule of bound inhibitor could reduce the digestibility of the major bonds cleaved by the three proteolytic enzymes used, steric shielding by the inhibitor as a cause of resistance against proteolysis could be excluded.

**MATERIALS AND METHODS**

Bovine pancreatic ribonuclease A, phosphate-free, chromato- graphically prepared, RAF 6059, was the product of Worthington. It was found pure by NH₂-terminal analysis (Table 1). Subtilisin was the subtilopeptidase A of Johansen and Ottosen (11), supplied as crystalline Alcalase, batch 50624, 25 Aæron units per g, by Novo Industri, Copenhagen, Denmark. Trypsin, twice crystallized, chymotrypsin (chromatographically homogenous), and soybean trypsin inhibitor, (pure, noncentrystallized) were obtained from Worthington. Yeast ribonuclease acid (lot 6232, Worthington) was used as substrate. 2'-G)'-Cytidylic acid was the product of P.L Biochemicals, and by spectrophotometric analysis (12) it contained 75% of the 2' and 25% of the 3' form. 2'-Cytidylic and 3'-cytidylic acids were isolated from a similar commercial sample by separation on a column of Dowex 1-X10, with formic acid elution (13), followed by repeated evaporation to remove formic acid, and were freeze-dried; they were shown to be pure as judged by their spectral properties (12). Potassium cyanate and urea (reagent grade), and thionine were from Fisher Scientific. N-Ethylmorpholine (Eastman Kodak) was redistilled.

**Digestion with Subtilisin**—A modification of the method of Gordillo, Vithayathil, and Richards (14) was used to carry out this digestion. The determination of the amount of ribonuclease-S formed was based on the finding of Allende and Richards (15) that trypsin rapidly inactivates the modified enzyme, while it has no effect of the intact ribonuclease. The coupled subtilisin-trypsin digestion was performed in the following way. Ribonuclease (1%) in 0.1 M sodium acetate buffer, pH 5.5, was digested by 0.01 to 0.03% subtilisin in an ice bath (solution temperature, 1-2°C). At appropriate intervals, 30 μl samples were removed and added to 30 μl of 0.1 M HC1. It was found that 1-hour incubation at room temperature at the resulting pH of 2.5 was sufficient to completely inactivate subtilisin. Following this, 20 μl of 0.1 M Tris buffer were added to bring the pH to about 8. Subsequently, 20 μl of 0.2% trypsin were added. Digestion for 1 hour at 25° was sufficient to inactivate all ribonuclease-S formed by subtilisin. After the addition of 4 ml of ice-cold 0.05 M acetate buffer, pH 5.0, the samples were ready for the determination of the amount of remaining active ribonuclease. It should be noted that neither acid treatment nor incubation with trypsin had any effect on ribonuclease A that had not been exposed to subtilisin. Furthermore, the presence of 2'-cytidylic acid modified only the rate of digestion by subtilisin; it did not protect against tryptic digestion, as expected from its very weak binding at pH 8, nor did it inhibit ribonuclease action in the final assay, in which it was diluted 25,000-fold with respect to its original concentration during subtilisin digestion.

**Digestion at 60° by Pancreatic Proteases**—The methods of Ooi et al. (9) and of Rupeil and Scheraga (10) were followed with some modifications.

(a) Trypsin—Ribonuclease A (1%) in 0.01 M KCl was digested at pH 5.5 for 15 min at 60° by 0.02% trypsin, at varying concentrations of inhibitors. At the end of the digestion, 30 μl samples were added to 3 ml of ice-cold 0.05 M acetate buffer, pH 5.0. Analyses of remaining activity were then carried out on these diluted samples.

(b) Chymotrypsin—The procedure differed from the one described above by the substitution of 0.01 M CaCl₂ for KCl. Chymotrypsin concentration was 0.05%.

**Determination of Ribonuclease Activity**—This was carried out according to the method of Shapiro (16), based on the rate of spectral shift undergone by thionine as it is released from its complex with ribonuclease acid in the course of digestion. The stock solution of thionine was 4.4 x 10⁻⁴ M, and it was kept in a dark bottle at 4°. Before use, it was diluted to 0.88 x 10⁻⁴ M with 0.05 M acetate buffer, pH 5.0. To 3.75 ml of this were added 25 μl of 5% RNA, and the solution was placed in an ice bath. Immediately before use, a portion of it was warmed to 28°. Aliquots of 1 ml were transferred to the cuvettes and were mixed with 5 or 10 μl of ribonuclease solution to be tested. Measurements in a Zeiss spectrophotometer were made at 625 μm against a blank containing 1.1 x 10⁻⁴ M thionine and no RNA. This resulted in an initial optical density of 0.7 to 0.9 of the RNA-containing samples in a 1-cm cell. The temperature of the samples was kept at 28° during the run by circulating water from a thermostated bath through the jacketed cell compartment. Optical density readings were taken every minute, until 0.3 optical density was reached. The resulting time curves were found to consist of two zero order segments. The optical density at which the break occurred was constant and independent of the rate of digestion. The slope of either segment could be used for comparison of rates in different samples. The fraction of active ribonuclease in the samples was calculated from the ratio of the slopes of digested and control samples.

The temperature-dependent transition of ribonuclease was measured by optical rotation in a Bredt-Ericsson polarimeter at 546 μm. A jacketed cell (length, 4 cm) was used. Ethylene glycol was circulated through the jacket from a thermostated bath. Five minutes were allowed for temperature equilibration at each temperature before the final reading was taken. Temperatures of the effluent solvent were monitored with a thermistor probe inserted into the rubber tubing at its junction with the jacket. The cell was checked for the presence of bubbles before and after each reading.
End Group Determinations—The carbamylation method of Stark and Smyth (17), modified to deal with 4 mg of protein in each case, was used. The incubation with cyanate was in 8 M urea, at 40°, for 14 to 16 hours. Analyses were made on the Beckman-Spinco amino acid analyzer, with the 53-em and 15-em columns. Care was taken to minimize destruction during the high temperature exposures. Metal-free “ultra-pure” HCl (E. Merck, Darmstadt, Germany) was employed in the cyclization, and for this and the hydrolysis, the solution was deoxygenated by bubbling washed nitrogen through it. For every sample a standard amino acid mixture (Beckman Instruments) was run for calibration. Correction factors for decomposition losses (17) were applied in the final calculations. NH₂-terminal residues were expressed on a molar basis, based on the amount of carbamylated ribonuclease separated in the procedure, measured by absorbance at 278 mp, with a molar absorptivity of about 0.5 residue per mole. All analyses were corrected for small amounts of glycine formed from cyanate alone during the carbamylation (17), which appeared as a spurious minor end group. A fraction of the samples (prior to alkaline hydrolysis) was therefore re-exposed to boiling HCl and passed through Dowex 50 (17) for the removal of this artifact. The correction thus determined, 0.1 to 0.18 residue per mole for all samples, was applied. An additional correction was needed for the small amount of glycine formed from cyanate alone during the carbamylation (17), which appeared as an apparent, low NH₂-terminal glycine level, since this glycine also became carbamylated. In dealing with intact proteins, this requires no correction, since in the procedure the reagents and other small molecules are removed on a Sephadex column in 50% acetic acid. However, in the case of enzymatic digests of protein, small fragments would be eluted in the same region, necessitating the inclusion of a region after the protein peak. To estimate the resulting contamination by carboxylglycine, native ribonuclease was analyzed both with and without inclusion of the corresponding post-protein zone in the Sephadex effluent fractions. The former sample showed no NH₂-terminal glycine present (Table I); the latter showed an apparent content of about 0.5 residue per mole. All analyses were corrected for this level of spurious glycine. Alanine, which can also form from glycine (17), was not significant. When the cyanate reaction was performed at 40°, instead of the recommended 50°, the glycine level was lowered, but complete carbamylatation was still permitted.

Results

Effect of 2′(3′)-Cytidylate on Rate of Formation of Ribonuclease–S—Fig. 1 shows the time course of the subtilisin-catalyzed formation of trypsin-sensitive ribonuclease, i.e. ribonuclease-S, at two concentrations of the protease. The experiment was carried out at 2°, as in the original method (6), and at pH 5.5. The latter was necessary in order to insure maximum binding of the inhibitor. Gordillo et al. (14) have shown that at pH 6 the yield of ribonuclease-S and the specificity of subtilisin action were the same as in the alkaline pH range. The data show that conversion to ribonuclease-S is incomplete, in agreement with the results of Gordillo et al. (14) who obtained yields not in excess of 60 to 80%. The presence of 2′(3′)-cytidylate, in an inhibitor to protein substrate ratio of 2:6, reduced the rate of ribonuclease-S formation to about 40% of the control rate. That subtilisin did act on the expected bond is shown by the results of end group analysis in Table I. For this determination, samples were digested by 0.05% subtilisin at pH 5.5 at 2°. After 60 min the digestion was arrested by lowering the pH to 2.5. As expected, both samples digested in the absence of nucleotide show serine as their major NH₂-terminal residue, originating mainly from the hydrolysis of the alanyseryl bond 20-21 (6), probably with some parallel cleavage at the serylseryl bond 21-22 (20, 21). It can be observed that in both of these samples, as well as in the ones digested in the presence of inhibitor, the yield of serine is higher than would be predicted on the basis of the amount of ribonuclease-S obtained. This high yield could be attributed to a combination of the following factors. (a) The notorious uncertainty in the serine determinations; the values given had to be corrected for a yield of 30% of NH₂-serine, determined by taking free serine through the entire end group analytical procedure. (b) The possibility that serine residues, other than those mentioned above, may be cleaved in this unusually serine-rich protein (12% of all residues) by subtilisin, a protease with a definite preference for seryl linkages in ribonuclease. (c) Finally, the possibility may be considered that in some molecules bond 20-21 is not attacked, but other serine linkages will be cleaved; this may result in the formation of active, trypsin-resistant ribonuclease, which will be assayed as intact ribonuclease. In addition, it may be observed that for unknown reasons the ratio of serine end group found to ribonuclease-S obtained is quite different in the two experiments (see Footnote i to Table I).

In addition to serine, small amounts of aspartic acid (or asparagine), threonine, glutamic acid (or glutamine), glycine, alanine, and valine have been found. This is not unexpected, since there are indications from the reports of those who have studied this process (at pH 8) that some other, slower cleavages proceed concurrently. Thus, Richards (22) found that on longer treatment about 30 peptide bonds in ribonuclease can finally be hydrolyzed by subtilisin, and Doscher and Hirs (20) note that, at the period of digestion when ribonuclease-S formation became maximal, approximately 20 ninhydrin-positive components were found in an analysis when the digested protein was plated directly on the column of the amino acid analyzer. These authors also found a maximum yield of ribonuclease-S of only 65 to 75%. With respect to the end groups additional to serine, found in the present study, it is worth noting that Richards and Vithayathil (6) found by qualitative dinitrophenylation that after the NH₂-terminal serine there appeared small quantities of NH₂-terminal aspartic or glutamic acid, alanine, and threonine in agreement with our results. It seems, therefore, that apart from the major attack at bond 20-21 (and 21-22) three other bonds are split slowly, but much faster than the many additional bonds that break under prolonged hydrolysis.

In the samples digested in the presence of inhibitor, the yield of NH₂-terminal serine is significantly reduced, in both experiments, as expected from the relative depression of ribonuclease-S formation. It may be observed that the presence of the nucleotide also decreased the extent of most of the minor cleavages, suggesting increased stability of the molecule at regions other than the critical one for ribonuclease-S formation. Thus, on this basis of both rate and end group analysis studies, we may conclude that the presence of the competitive inhibitor in the
active center decreases the rate of cleavage of one or more bonds situated outside of this region.

Effect of 2'- and 3'-Cytidylate on Rate of Tryptic and Chymotryptic Digestion of Ribonuclease—Fig. 2 shows the extent of inactivation of ribonuclease when digested by trypsin and by chymotrypsin for 15 min at 60°C as a function of the concentrations of the two nucleotides. In the studies of Rupley and Scheeraga (10) and of Ooi et al. (9), digestions were carried out at pH 6.5; in the present case, as mentioned earlier, digestions were done at pH 5.4 in order to insure maximum binding of the inhibitors. To compensate for low proteolytic activities at this low pH, we had to use 10 times the amount of chymotrypsin and 50 times the amount of trypsin that was used in the aforementioned studies. This was also necessitated by the somewhat greater heat stability of our ribonuclease preparation as compared with theirs (see below). In the absence of inhibitors, trypsin and chymotrypsin destroyed 90 and 62% of the original activity, respectively. Of the two inhibitors, 2'-cytidylate proved much more effective in retarding the rate of inactivation, as expected from its greater affinity for the enzyme. It can be seen that maximum protection by 2'-cytidylate against tryptic attack occurs at about 0.75 × 10^{-3} M cytidylate, which corresponds to a 1:1 molar ratio of enzyme to inhibitor. Pyrophosphate was slightly more effective against the action of chymotrypsin than was 3'-cytidylate; 3'-adenosine monophosphate was completely inactive.

The cleavages by chymotrypsin at 60°C found in ribonuclease A digested in the absence of inhibitor (Table I) agree well with those found by Rupley and Scheeraga (10). The amounts of the three additional NH_{2}-terminal groups are small, and since these authors only examined the major, high molecular weight products separated by Amberlite IRC-50 chromatography, their data do not exclude the occurrence of these minor cleavages elsewhere.

In the case of trypsin, acting at 60°C, our data differ somewhat

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**TABLE I**

**NH_{2}-terminal amino acids in ribonuclease digests**

All NH_{2}-terminal residues detectable (above 0.02 residue per mole) are shown. NH_{2}-terminal cysteine was not measured. All figures represent the means of at least two sets of separate end group determinations done on the same sample. Serine is only quoted to the nearest 0.1 residue, on account of the lower accuracy due to the large correction for decomposition (17). For similar reasons, threonine is to the nearest 0.05 residue.

<table>
<thead>
<tr>
<th>End group</th>
<th>Treatment of ribonuclease</th>
<th>Subtilisin(^a)</th>
<th>Trypsin(^b)</th>
<th>Chymotrypsin(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment I</td>
<td>Experiment II</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No inhibitor</td>
<td>2'(3')-cytidylate(^d)</td>
<td>No inhibitor</td>
<td>2'(3')-cytidylate(^d)</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.88</td>
<td>0.60</td>
<td>0.66</td>
<td>0.84</td>
</tr>
<tr>
<td>Aspartic acid or asparagine</td>
<td>0</td>
<td>0.14</td>
<td>0.06</td>
<td>0.19</td>
</tr>
<tr>
<td>Threonine</td>
<td>0</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Serine</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.12</td>
</tr>
<tr>
<td>Glutamic acid or glutamine</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
<td>0.10</td>
</tr>
<tr>
<td>Glycine</td>
<td>0</td>
<td>0.11</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>Alanine</td>
<td>0</td>
<td>0.03</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Valine</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sum of new end groups</td>
<td>0</td>
<td>1.48</td>
<td>0.77</td>
<td>1.21</td>
</tr>
<tr>
<td>Ribonuclease-S (%) formed or inactivation (%)</td>
<td>0</td>
<td>32(^h)</td>
<td>11</td>
<td>47(^i)</td>
</tr>
</tbody>
</table>

\(^{a}\) Subtilisin (0.033%) for 60 min at 2°C. Digestion terminated by adjustment of pH to 2.5.

\(^{b}\) Trypsin (0.025%) for 15 min at 60°C. Digestion terminated by addition of soybean trypsin inhibitor.

\(^{c}\) Chymotrypsin (0.05%) for 15 min at 60°C. Digestion terminated by placing the tube in boiling water for 10 min.

\(^{d}\) 2'-Cytidylate (75%) and 3'-cytidylate (25%) present during digestion at 2.6 times the molar concentration of ribonuclease.

\(^{e}\) These two independent experiments were performed identically. Variations in the amount of ribonuclease-S formed have been repeatedly observed. The samples digested in the presence of inhibitor, however, always showed a marked decrease in the yield of ribonuclease-S.

\(^{h}\) Position unknown.

\(^{i}\) These columns list the sequence positions of amino acids found by previous workers to be NH_{2}-terminal upon similar treatment of ribonuclease. See Reference 9 for trypsin and Reference 10 for chymotrypsin.

\(^{i}\) Small amounts of a serine end group are often found in native ribonuclease. The value of 0.1 was subtracted from all serine end groups reported.

\(^{i}\) This residue is variable in yield, to an extent outside the experimental error in the cyanate method; further repeats of the complete analysis have been made, and in four determinations the yield increased from 0.3 to 1.1 groups per molecule, as the sample was stored in the frozen state. This variability is probably due to the formation of pyrohydroxycarboxylic acid from the NH_{2}-terminal glutamic acid (or glutamine) produced, a phenomenon observed previously (18, 19) to occur readily in a peptide with NH_{2}-terminal glutamine produced in a complete tryptic digestion of oxidized ribonuclease. The residue in that study was glutamine 11. The analyses on our tryptic digest were made after varying periods of storage in the frozen state, and it is possible that such storage, or the freezing and thawing, increases the yield of the cyclized form.
from those obtained by Ooi et al. (9). The cleavages at bonds 31-32 and 33-34 found by these authors also occur completely in our digestion. However, we find a significant amount of a new NH₂-terminal group, glutamic acid or glutamine (see Footnote g to Table I). There are three positions in the ribonuclease sequence where glutamic acid, or its amide, occurs on the COOH-terminal side of lysine or arginine; these are residues 2, 11, and 86. Bond 1-2 which contains the nonacylated, NH₂-terminal lysine can be excluded, since it is never split by trypsin (23). However, a connection between the presence of the new NH₂-terminal glutamic residue and the low yield of NH₂-terminal lysine in this analysis can be suggested. It is noted that this is the only case in which we found the NH₂-terminal lysine in unusually low amounts. If bond 10-11 was split to produce the glutamic end group, the resulting peptide fragment would be significantly separated from the protein component in the ge filtration step, and this would result in a loss of the lysine NH₂-terminal group. We suggest therefore that probably some cleavage, additional to those reported (9), occurs in our digestion with trypsin. It seems likely that this involves the arginylglutaminyl bond 10-11. The split at this bond seems to be a major one. That it was not found in the studies of Ooi et al. (9) may be due to the lower pH used by us. If indeed, bond 10-11 is involved, the adjacent residue, histidine 12, will change its state of ionization in just this pH range (24), perhaps inducing a local conformational change.

The results of NH₂-terminal analysis thus show that the bonds cleaved by the two pancreatic proteases are, in all likelihood, the ones identified in the studies quoted above (9, 10). Since in those studies it was shown that the fragments obtained were either completely inactive, or very weakly active, loss of activity must be a direct consequence of the hydrolysis of these bonds. Preservation of activity in the presence of the nucleotides, therefore, implies that these bonds must have been protected against proteolytic attack. Inasmuch as a single nucleotide could not have afforded steric shielding to some nine peptide linkages, we must conclude that the presence of the inhibitor in the active center resulted in a conformational change which expressed itself as increased resistance against proteolytic attack, observable at nine different loci along the ribonuclease sequence.

**Effect of 2'-Cytidylate on Temperature-induced Transition of Ribonuclease**—The decreased resistance of ribonuclease to proteolytic attack at high temperatures is only one manifestation of the cyanate method has the advantage for this kind of work of being more accurate than the methods which employ paper chromatography, by virtue of its greater sensitivity which permits detection of end groups liberated at the initial stages of proteolysis. However, it has the disadvantage that it is difficult to determine the NH₂-terminal groups of small peptides and amino acids when large peptides are analyzed in the same mixture. As mentioned above, this is due to the necessity of removing urea and cyanate on a Sephadex column following the carbamylation step, and thereby also removing small peptide material. A degree of uniformity was introduced into the present results by taking the cuts in all samples at the same stage of urea elution. A modification involving a separate chromatographic separation of all fractions is needed and should be readily applicable.
of the temperature-induced conformational transition discovered by Harrington and Scheffman to occur in this molecule (25). The transition is evidenced by changes in optical rotation (25, 26), ultraviolet absorption (26), and viscosity (27), and has been interpreted as a cooperative unfolding of the structure with increasing temperature (25). It was of interest, therefore, to see whether, in the presence of 2'-cytidylate in the active center, the transition would be displaced to higher temperatures. Fig. 3 shows the specific rotation of ribonuclease at 546 μm, measured between 45 and 80°, in the presence and absence of the inhibitor. 

The transition in the control curve has the expected slope, but the midpoint of the transition is at 67°, 4 degrees higher than the value obtained at pH 5.5 by Hermans and Scheraga (26). Since the preparation used was claimed by the manufacturer to be phosphate-free, the reason for this apparent stabilization is not known. The effect of the nucleotide was unexpectedly strong; not even at 80° was there any sign of the transition. The actual value of this constant specific levorotation is about 12% higher than the low temperature level of the control curve. This is in agreement with the results of Cathou, Hamnes, and Schimmel (28) who measured the optical rotatory dispersion of ribonuclease in the presence of 2'-cytidylate. The differences due to 
in the presence of the inhibitor were most pronounced in the 228 mμ region and were interpreted as an indication of a conformational change in the enzyme caused by the interaction, and not as a contribution from either the free or the bound nucleotide. 

The high degree of stability of the optical rotation in the presence of the inhibitor is in apparent contradiction to the results of Hammel, Ver Ploeg, and Nelson (29) who found considerable differences between the active center of ribonuclease in the presence of 2'-cytidylate. The differences due to 
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The high degree of stability of the optical rotation in the presence of the inhibitor is in apparent contradiction to the results of Hammel, Ver Ploeg, and Nelson (29) who found considerable temperature dependence at pH 5.5 of the 2'-cytidylate induced absorbance difference at 260 mμ in ribonuclease A. When the dissociation constant of the complex was calculated from the absorbance difference at several temperatures, these authors obtained a heat of dissociation of 20,800 cal per mole. In contrast, our results suggest that the free energy of interaction between the inhibitor and ribonuclease is supplied entirely by the entropy change. Alternatively, one may assume that at high temperatures the nucleotide is readily dissociated, but the unfolding of the structure from the conformation previously stabilized by the inhibitor is extremely sluggish.

DISCUSSION

The finding that substrate analogues can stabilize the structure of ribonuclease is not in itself a new one. Resistance to denaturation by 8 M urea has been obtained in the presence of mono- and polyphosphates (30-33), some di-anions (31, 27), and nucleotides (31, 33). Similarly, increased resistance against thermal unfolding has been observed with sulfate and phosphate ions (27, 32). Data on decreased proteolytic digestibility are far fewer in the literature. However, Ginsburg and Carroll (27) observed decreased digestibility of ribonuclease by pepsin at 50° in the presence of sulfate but not in the presence of chloride, and Rupley and Scheraga (34) found greatly reduced chymotryptic digestion rates in the presence of sodium phosphate and citrate.

The significance of the present study lies in the fact that the exact location of both effector and effect is known, and the ambiguities of interpretation are thereby greatly reduced. This was assured by the choice of 2'-cytidylate as the ligand, an agent known to bind specifically, stoichiometrically, and in a 1:1 ratio, to the active center of ribonuclease, at pH 5.5 (29, 35), and by the choice of points of proteolytic attack, the exact location of which had been accurately determined by past work in this field. We feel that the results obtained under these conditions now prove that the presence of a small molecular ligand in the active center provides increased resistance against disruptive influences in regions of the molecules that are remote from the binding site, and are, in fact, distributed over the entire protein molecule. Steric shielding of a susceptible bond by the ligand, at least in this case, can thus no longer be invoked to explain decreased proteolytic rates. By inference, the data also provide evidence that the conformational rigidity of ribonuclease in at least nine defined regions is altered in the presence of ligand with respect to the time average conformation that prevails when the active center is unoccupied. The three-dimensional structure of ribonuclease, recently elucidated by Kartha, Bello, and Harker (36) and by Wyckoff et al. (37), shows that none of the identified bonds cleaved by the three proteases used in this study are in the immediate vicinity of the active center, as marked by the location of the phosphate group and by that of histidines 12 and 119. The subtilisin-sensitive bond 20-21, in particular, is located at the opposite side of the molecule.

It should be noted that in the study to which we have just referred (30), crystals of ribonuclease could be obtained only in the presence of phosphate, sulfate, arsenate, or citrate, i.e. ions known to stabilize ribonuclease, and that 1 molecule of these ions was found to be present in the crystals, in the region assigned to the active center. This in itself can be taken as evidence that the ions reduced the conformational fluctuation in the molecule, thereby permitting a degree of conformational homogeneity compatible with the regular packing of molecules in the crystal lattice. It would follow from this that the published structure is that of the ligand-stabilized form. It is interesting to note that, since bond 20-21 appears to be completely exposed even in this, presumably stabilized structure, it would seem that in addition to exposure to the solvent, "molitility" of the peptide segment to be attacked is also necessary for efficient proteolysis, as suggested in an earlier publication (2).

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