Studies on the Conformation and Proteolytic Susceptibility of Rat and Bovine Pancreatic Ribonucleases

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SUMMARY

The amino acid sequence of rat pancreatic ribonuclease has been found by Beintema and Gruber to differ from that of the bovine enzyme in approximately one-third of the residues. We have found that, in spite of this large number of amino acid replacements, the two proteins are very similar in their over-all conformation as attested by optical rotatory dispersion and circular dichroism in the far ultraviolet region of the spectrum. Some local differences in conformation have been detected by the use of proteases which show that the subtilisin- and elastase-sensitive region of the bovine enzyme is altered in the rat enzyme. Circular dichroism studies in the far ultraviolet region indicate that the exposed tyrosine residues of the bovine enzyme contribute significantly to the optical activity of the protein in the near ultraviolet spectral region.

Although bovine pancreatic ribonuclease is one of the most thoroughly studied of proteins, the corresponding enzymes from other species are less well characterized. In recent years, there has been considerable interest in the comparative biochemistry of this enzyme, and partial or complete amino acid sequences have been derived for a number of pancreatic ribonucleases. Jackson, Reinhold, and Hirs (1) have recently reported much progress in their structural studies of the porcine enzyme, and Beintema and Gruber (2) have deduced partial amino acid sequences for the horse enzyme as well as the complete amino acid sequence of rat ribonuclease. As an accompaniment to this structural work, a number of laboratories have studied the specificity and other functional properties of pancreatic ribonucleases from various species (3-9). These enzymological studies have shown that a close similarity exists in the catalytic properties of the enzyme from different mammalian species which is in contrast to the sometimes very large number of amino acid replacements found in the structural studies.

The amino acid sequence of rat ribonuclease, as determined by Beintema and Gruber (2), shows this enzyme to contain more than 40 differences from the bovine enzyme in a total of fewer than 130 amino acid residues. Nevertheless, all amino acid residues which are known to be important in the maintenance of the structure or function of the bovine enzyme are present in the appropriate position of the rat enzyme (2). The present work was initiated with the aim of elucidating the effects, on the conformational properties of ribonuclease, of the large number of amino acid replacements observed in these two species. Our studies have centered on the over-all conformation, as measured by far ultraviolet optical rotation, and on the structural properties of some small and defined areas of the enzyme, as measured by proteolytic susceptibility and optical rotation in the near ultraviolet region.

MATERIALS AND METHODS

Circular Dichroism and Optical Rotatory Dispersion—These measurements were performed with a Cary 60 spectropolarimeter at 26° using techniques previously described (10). The data are expressed as mean residue ellipticity [θ] or as reduced mean residue rotation [θ] defined as usual (11, 12). The mean residue weight of both bovine and rat RNase is 110.

Ultracentrifugation—A Spinco model E ultracentrifuge equipped with the photoelectric scanner, with a monochromatic light at 280 nm, was used. A partial specific volume of 0.695 (13) was assumed for both species of enzyme.

Proteolysis Experiments—These were carried out in the Radiometer pH-stat apparatus as previously described (14). The reaction mixtures were 1 ml in volume, and temperature was controlled generally to 25° ± 0.1°. The burette used was a 50-μl Hamilton syringe which enabled accurate measurements of base addition in the microliter range. RNase concentrations studied were uniformly 1 mg per ml in 0.1 M NaCl at pH 8. The amounts of protease used varied with the experiment from 10 to 100 μg.

Discontinuous Polyacrylamide Gel Electrophoresis—This was carried out with the pH 4.5 system of Reisfeld, Lewis, and Williams (15). The proteins were stained with Amido Schwartz in the usual manner.

Ion Exchangers—The ion exchangers used in this work were Amberlite IRC-50 (XE-64), 200 to 400 mesh, from the Rohm and Haas Company, Philadelphia, Pennsylvania, purified as described by Hirs, Stein and Moore (16), sulfoethyl-Sephadex C-25 from Pharmacia, and carboxymethylcellulose, a product of Serva (containing 0.5 meq capacity per g).

Ribonuclease Assays—These assays were performed at pH 5, with yeast RNA used as substrate in accordance with the precipitation method previously described (17).

Trypsin was obtained from Worthington and was either a twice crystallized preparation or an L-(1-tosylamido-2-phenyl)-1227
Ethyl chloromethyl ketone-treated preparation. Crystalline porcine elastase was also a Worthington product. Subtilisin (Nagarse) was obtained from the Biddle Sawyer Company, New York, New York. Bovine pancreatic ribonuclease A was Sigma type XII.

**Rat Pancreatic RNase**—A typical preparation of rat pancreatic RNase will be described in some detail since the procedure which we used is significantly different from those reported by earlier workers (6, 18). A 217-g sample of frozen Sprague-Dawley rat pancreas was homogenized together with 65.0 ml of 0.25 M H$_2$SO$_4$ in a Waring Blendor at 2°C for three 2-min periods and one 1-min period, with 10 min allowed for cooling between each homogenization. The homogenate was then stirred at 2°C for 48 hours, and clarified by centrifugation at 30,000 rpm for 30 min in the IECSO preparative ultracentrifuge, at 5°C. The layer of fat which is formed in this process was removed with a spatula, and the slightly cloudy supernatant fluid was removed by decantation. After the pH of this solution was adjusted, at room temperature, to 5.8 with 1 N NaOH, the volume was 800 ml. Then, 312 g of solid ammonium sulfate (60% saturation) were added slowly with continuous stirring, again at room temperature. After an additional 30 min of stirring, the precipitate was removed by centrifugation at 30,000 rpm for 20 min. The supernatant fluid was filtered through glass wool to remove a few remaining fat particles, and emerged as a clear, light yellow liquid. This filtrate was brought to 100% saturation with ammonium sulfate by suspending 50.8 g of the solid salt in an 18/32 dialysis membrane in the pooled fractions prior to centrifugation. The cloudy solution was clarified by centrifugation at 30,000 rpm for 20 min. The cylinder and the outside of the bag were washed with saturated ammonium sulfate, and these washings were added to the original solution prior to centrifugation. The slightly yellow precipitate was dissolved in 7 ml of water and dialyzed for 6 hours against two changes of 3 liters of water in an 18/32 dialysis casing. The sample was applied to a XE-64 column (19.6 × 84 cm) which was equilibrated and eluted with 0.2 M sodium phosphate buffer at pH 6.35 (16). Fractions of 4.7 ml were collected and monitored at 280 nm (--) as well as by RNase assay (---) as shown. The major active component was pooled as shown by the heavy line (--), and was further purified, as described under "Materials and Methods."

**TABLE I**

**Amino acid analysis of rat ribonuclease**

| Residues/mole protein | This work$^a$ | Literature$^b$ | Theoretical$^c$ |
|-----------------------|--------------|----------------|----------------|
| Lysine                | 11.0         | 10.0           | 11             |
| Histidine             | 5.0          | 4.8            | 5              |
| Arginine              | 6.0          | 6.0            | 6              |
| Aspartic acid         | 15.7         | 15.4           | 15             |
| Threonine             | 10.0         | 10.7           | 10             |
| Serine                | 10.5         | 15.2           | 15             |
| Glutamic acid         | 12.3         | 12.4           | 12             |
| Proline               | 7.2          | 6.7            | 7              |
| Glycine               | 8.2          | 8.6            | 8              |
| Alanine               | 4.6          | 4.0            | 4              |
| Half-cystine          | 7.8          | 7.1            | 8              |
| Valine                | 6.5          | 6.7            | 7              |
| Methionine            | 3.0          | 3.2            | 4              |
| Isoleucine            | 4.5          | 3.9            | 5              |
| Leucine               | 3.2          | 3.0            | 3              |
| Tyrosine              | 3.7          | 3.8            | 4              |
| Phenylalanine         | 3.2          | 3.1            | 3              |
| Glucosamine           | 0            | 0              | 0              |

$^a$ The sample was hydrolyzed in 5.7 N HCl for 18 hours at 110°C in a nitrogen atmosphere. The data are uncorrected for losses.

$^b$ Hydrolysis for 20 hours, also uncorrected for hydrolysis losses (18).

$^c$ These are the values required by the sequence as found by Beintema and Gruber (2).
overnight with stirring. The resulting suspension was centrifuged as before (along with washings), and the precipitate was dissolved in 2 ml of 0.1 M NaPO₄, pH 6.58, and dialyzed for 6 hours against three changes of the same buffer. The sample was then applied to a column (1 × 38 cm) of sulfoethyl-Sephadex C-25 (Pharmacia) which had been equilibrated and was eluted with the same 0.1 M NaPO₄, pH 6.58, buffer (19). Fractions, 2.5 ml each, were collected at a rate of about one per hour. A number of protein peaks are seen in the elution diagram (Fig. 2). The major portion of the ribonuclease activity is associated with the most abundant protein component which emerges near Fraction 40. This material (Fractions 38 to 45) was combined, diluted with 1 volume (20 ml) of water, and the pH adjusted to 5.0 with glacial acetic acid. The sample was applied to a column (2.5 × 8.5 cm) of carboxymethylcellulose (adjusted to pH 5 and washed well with water), and the column was washed with 250 ml of water to remove the bulk of the salts. The RNase was eluted with 0.4 M NH₄HCO₃, pH 8.0, and the effluent fluid was collected in fractions of 5 ml each. The enzyme was concentrated in Fractions 6, 7, and 8 which were pooled and lyophilized. Final desalting was achieved by passing this material, dissolved in 2 ml of H₂O, through a column containing 4 ml of a mixed bed ion exchange resin (Bio-Rad AG501-X8D). The column was washed with 25 ml of water, and the effluent was lyophilized to yield 24.7 mg of rat ribonuclease.

RESULTS

Characterization of Rat Ribonuclease—The amino acid composition of the rat ribonuclease which we prepared is shown in Table I. The results are expressed as residues of amino acid per mole of protein and have not been corrected for losses due to destruction or incomplete hydrolysis. A similar set of data, obtained from the work of Beintema (18) is included for comparison, as are the theoretical values. It is clear from the table that our preparation gives an amino acid analysis which is in good agreement with that of Beintema and with the theoretical expectations based on the amino acid sequence. The low values of serine which we found can easily be attributed to losses during
hydrolysis. The methionine value is low both in our analysis and in that of Beintema for unknown reasons. The complete absence of glucosamine from the enzyme is in accord with the finding of Beintema and Gruber that the rat enzyme does not contain carbohydrate. This is in contrast to the equine (2) and porcine (1, 20) enzymes, both of which are glycoproteins.

It may be noted that rat ribonuclease contains only 4 tyrosine residues per mole in place of the six found in the bovine enzyme. Since neither species of enzyme contains tryptophan this should result in a marked decrease in absorbance in the near ultraviolet region. Fig. 3 shows that this is indeed the case and that the absorbance of the rat enzyme near 280 nm is approximately two-thirds that of the bovine enzyme, as was expected (4). The rat enzyme which we have prepared migrates as a homogeneous species on disc gel electrophoresis (6) and is clearly distinguishable from the bovine enzyme by this technique, as is shown in Fig. 4. The rat enzyme also behaves as a single species in the analytical ultracentrifuge; it migrates with a rate corresponding to an \( s_{20,w} \) of 1.85. A molecular weight determination of the rat enzyme by high speed sedimentation equilibrium showed that the enzyme behaves as a single, monomeric species with a molecular weight of 14,850 (Fig. 5). The activity of the rat enzyme
Fig. 10. A comparison of the amino acid sequences of bovine (40, 41) and rat ribonuclease (2). The numbering system adopted for the rat enzyme is chosen to demonstrate the high degree of homology of the two sequences. Underlined residues are those which differ in the two sequences. The large hydrophobic residues are in capital letters (18).

measured at pH 5 with RNA as substrate, is only slightly lower than that of the bovine enzyme on a weight basis (Fig. 6). These results all attest to the high degree of homogeneity of our preparation and are in accord with the data of Bemtema and Gruber (14), Gordon (15), and Cozzone and Marchis-Mouren (16) on the general properties of rat pancreatic RNase.

Conformational Studies—Our principal purpose in this investigation was to assess the effect of the large number of amino acid replacements in the rat enzyme on the conformation of the molecule. We therefore examined the optical rotatory dispersion of the two RNases (Fig. 7) as well as their circular dichroism spectra (Fig. 8). The two types of measurement give essentially the same results, although in different form. It is useful to focus attention first on the curves below 240 nm since these are most easily correlated with the over-all molecular conformation. The rotatory dispersion curve of the bovine enzyme is in good agreement with those already published (21-24) and shows the well known trough near 228 nm which is characteristic of this protein and not commonly observed among other protein optical rotatory dispersion curves. It is therefore significant that the rat enzyme displays this same minimum near 228 nm in the optical rotatory dispersion curve. There is a small difference in the amplitude of this trough which is not far above the experimental uncertainty of the measurements. The circular dichroism curves of the two enzymes in the far ultraviolet region are also very similar both in shape and magnitude. Here again the shape of the RNase curve (22-25) is characteristic and highly unusual and is thought to reflect the particular mixture of helix, \( \beta \) structure, and more random type structure believed to be present in the RNase molecule (22). These results suggest that the rat enzyme contains these structures in the same proportions and that the gross conformation of the two species of RNase is similar.

The near ultraviolet optical rotatory dispersion and circular dichroism curves of the two enzymes (inset, Figs. 7 and 8) show that the Cotton effect centered about 275 nm is much diminished in the rat enzyme when compared to the bovine enzyme. This difference apparently reflects the loss of 2 tyrosine residues, numbers 73 and 76, in the rat enzyme. The 6 tyrosine residues of the bovine enzyme are divided into two classes of 3 residues each. One class titrates normally, and is readily iodinated. This class, comprising Residues 73, 76, and 115 (26, 27) is now known to be at least partly exposed to the solvent (28, 29). The second class does not normally titrate below pH 12, is resistant to iodination, and is relatively buried in hydrophobic regions of the molecule. Rat ribonuclease has an unchanged complement of buried residues, but is missing 2 of the 3 exposed tyrosines (4). There has been a good deal of controversy about the contribution of these two classes of tyrosine residue of bovine RNase to the near ultraviolet Cotton effect. The work of Dezychok (11) and Simmons and Glazer (30), which showed a shift in the wave length of the tyrosine Cotton effect with pH in the region of titration of the exposed tyrosines, led to the conclusion that this class of residues is primarily responsible for the “extrinsic” Cotton effect. On the other hand, the work of Simpson and Vallec (31) and Beaver and Gratzel (32), in which some tyrosine residues were preferentially modified by acetylation or by nitrosonium, without markedly affecting the tyrosine Cotton effects of bovine RNase, led to the opposite conclusion, namely, that it is the buried tyrosine residues which are primarily responsible for the Cotton effect. Our present studies indicate that Residues 73 and 76 contribute something close to 50% of the total Cotton effect as measured by circular dichroism.1 We conclude that at least part of the tyrosine Cotton effect of bovine RNase can be ascribed to exposed tyrosine residues.

Proteolysis—The sensitivity of bovine RNase to proteolysis has been studied extensively. It is known that the bovine enzyme in its native state is resistant to the action of trypsin and chymotrypsin (33, 34), amionopeptidase (35), and carboxypeptidase (36), but that the peptide bonds in the region between alanine Residue 19 and serine Residue 22 are particularly susceptible to the action of subtilisin (peptide bonds 20 and 21) (37-39) and pancreatic elastase (peptide bonds 19 and 20) (40, 41) (41). Accordingly, we have studied the action of these two enzymes on native rat RNase. A typical set of experiments is shown in Fig. 9. The figure shows the base uptake at pH 8, as a function of time, as a measure of the rate of peptide bond breakage catalyzed by the proteases. It is seen that although the conditions of the experiment are such as to produce rapid hydrolysis of bovine RNase by both subtilisin and elastase, the rat enzyme is completely unaffected. Thus, the proteolytic susceptibility of the region near Residue 20 of the rat enzyme is reduced to an undetectable level. Since alanine 20 of the bovine enzyme is replaced by lysine in the rat enzyme (Fig. 10), we made a number of attempts to demonstrate a susceptibility of the native rat enzyme to trypsin. These attempts were uniformly unsuccessful. Chromatographic analysis of rat RNase after exposure to trypsin, subtilisin, or elastase showed only a single peak corresponding to the native enzyme, and thus confirms the pH-stat data. Thus, the resistance of this region of the rat enzyme to proteolysis is not simply the result of the lack of appropriate side chains with which to satisfy the specificity requirements of the proteases, but is apparently the result of a conformational alteration.

**DISCUSSION**

It is useful, in considering the results of this study, to be able to refer to the amino acid replacements in the rat enzyme as compared with the bovine. We have, accordingly, presented the two amino acid sequences in a parallel fashion in Fig. 10.

A detailed comparison of the two sequences has been made by Wyckoff (42). He concluded that even though more than 30% of the amino acid residues of rat RNase are different from those of the bovine enzyme, there is little reason to expect that large conformational alterations have resulted, since the rat enzyme

1 This conclusion rests on the assumption that the contribution to the circular dichroism and optical rotatory dispersion spectra of the 4 invariant tyrosine residues is closely similar in the two enzymes. In view of the over-all similarity in conformational and other properties of the two proteins, this assumption would appear to be justified.
sequence can readily be accommodated into the crystal structure of the bovine enzyme. The circular dichroism and rotatory dispersion data which we have obtained confirms this expectation. The most straightforward interpretation of our results is that the amounts of ordered structure, that is, α-helix and cross β structure, are the same in the 2 molecules, and that as a result of this equivalence, the conformations of the two must be grossly similar.

It does not follow, of course, that the structure of the less regularly organized regions of the molecule are also the same. The lack of susceptibility of the rat enzyme to subtilisin, elastase, and trypsin shows that the structures of the 2 molecules probably differ greatly in the region between Residues 18 to 23. This region of the molecule is also one which contains a very high proportion of amino acid replacements, including two of the three serine-proline interchanges. Peptide bonds adjacent to proline are sometimes resistant to proteolysis (43), so that the proline-serine bond at Residues 18 and 19 and the serine-proline bond at Residues 22 and 23 might be resistant to attack by the usual proteases. On the other hand, peptide bonds once removed from proline residues are normally susceptible to proteolysis (44). Indeed, Segal, Himmelharp, and Harrington (45) have found that the region of the myosin molecule which is particularly susceptible to proteolysis is relatively rich in proline, and Smyth and Utsumi (46) have made a similar observation with the protease-sensitive region of rabbit immunoglobins. In analogy with these observations, the 18 to 23 residue stretch of rat RNase should be particularly easily attacked by proteases. Thus, the explanation for the lack of susceptibility of the rat enzyme to subtilisin, elastase, and trypsin must be in the changed conformation of this region of the molecule with respect to that of the bovine enzyme. There is little reason to suppose that the local conformation of this region of the rat enzyme is more highly ordered than is the corresponding region of the bovine enzyme. Indeed, the two prolines in the rat enzyme would most likely preclude any regular type of structure. The balance of probabilities would, therefore, suggest that it is the presence of a particular type of structure in the bovine enzyme that leads to the pronounced proteolytic susceptibility of this region and that this structure is not found in rat RNase.

Kartha, Bello, and Harker (28) have shown, on the basis of x-ray crystallography, that alanine Residues 19 and 20 of bovine pancreatic ribonuclease A form a sharp bend in the polypeptide chain which protrudes somewhat from the rest of the molecule. This feature of the bovine enzyme can be the cause of the proteolytic sensitivity of this region and is similar to a structure which had previously been suggested to account for the proteolysis data (14). Our present experiments suggest that this type of protruding structure will not be found in rat ribonuclease.

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