SOME SPECTROPHOTOMETRIC AND POLARIMETRIC EXPERIMENTS WITH RIBONUCLEASE

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INTRODUCTION

Previous studies on the activity and physical properties of ribonuclease in strong urea solutions[1,2], indicate that its enzymic function is unimpaired in spite of what appears to be extensive disruption of the hydrogen bonded tertiary structure. Furthermore, it has been found that limited proteolysis by both subtilisin[3,4] and by carboxypeptidase[5] leads to the formation of derivatives possessing full activity. One may conclude, therefore, that the complete, integrated structure of this enzyme is not essential for the expression of its catalytic properties.

The converse of this conclusion has been reached from experiments on the limited digestion of ribonuclease with pepsin[6,7]. Thus, following very short exposure of ribonuclease to pepsin at pH 1.8, a derivative may be isolated which is totally inactive and which appears to differ from native RNase only by the absence of the four amino acid residues constituting the C-terminal end of the ribonuclease chain. Restricted cleavage by pepsin has also been observed for ACTH[8] and for diphtheria antitoxin[9]. Further findings supporting the conclusion that the early pepsin-induced changes in ribonuclease are indeed restricted to a specific peptide bond are presented below.

The present report deals with spectrophotometric and polarimetric experiments on native RNase and pepsin-inactivated ribonuclease (PIR) in which an attempt has been made to establish the presence or absence of correlation between various tertiary structural aspects of the enzyme and its enzymically active "center".

MATERIALS AND METHODS

Bovine pancreatic ribonuclease (Armour, Lot No. 381–059) and pepsin (Worthington, Lot No. PM 522) were used throughout. The RNase contained 9.38 % water. U.V. absorption measurements were made at approximately 25° on a Cary Recording Spectrophotometer, Model 11 MS. The extinction coefficients, ε, were calculated assuming M.W. 13,683[10], and correcting for water content. Optical rotation measurements were made, at approximately 25°, on a Rudolph Photoelectric Polarimeter Model 200. The optical rotation changed, in all cases, linearly with concentration in the range studied (c = 6–50 g/l). Enzyme activity was determined as described previously[11].

RESULTS

Preparation and further characterization of PIR

RNase was briefly digested with pepsin as previously described[6,7] and the inactive product isolated by chromatography on IRC-50 ion exchange columns. After sepa-

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ration of the derivative from the buffer salts by precipitation from a concentrated solution with 9 volumes of acid–acetone (1 part 1 N HCl: 39 parts absolute acetone), aliquots were treated with 6 times recrystallized DFP-treated carboxypeptidase[12] (kindly given by Dr. J. A. GLADNER). Carboxypeptidase was used at a level of 1 mole/25 moles of PIR. The reaction was followed by chromatographic analysis of dinitrophenylated[13] samples of the reaction mixture taken at 15 and 120 minutes. After 120 minutes, the yield of phenylalanine was 40%. Traces of a number of other DNP amino acids were detected, but their total amount was only one-third that of DNP-phenylalanine. Only DNP-phenylalanine was detected by this procedure in the early sample. The presence of this amino acid at the C-terminal end of the PIR chain is in agreement with the earlier observation that a tetrapeptide, Asp–Ala–Ser–Val, is removed during the limited pepsin action[7]. The present results suggest, incidentally, that this phenylalanine residue is not preceded by a proline residue, since the presence of a penultimate proline residue generally inhibits completely. Qualitative confirmation of the exclusive presence of C-terminal phenylalanine was obtained by treatment of PIR with hydrazine according to NIU AND FRAENKEL-CONRAT[14].

Spectrophotometric changes during proteolysis

SHUGAR[15] has shown that there occurs a shift in the tyrosine region of the U.V. spectrum of ribonuclease during pepsin digestion. An extension of these studies, in which loss in the progressive enzyme activity has been correlated with the magnitude of the shift, is summarized in Figs. 1, 2, and 3. In Fig. 1 are shown spectra of RNase and PIR obtained before and after digestion. Native RNase, at pH 6.5 and 25° C has an absorption maximum at 2775 A (ε = 9800). "Difference" spectra, comparing control solutions of native RNase with various experimental solutions, are shown in

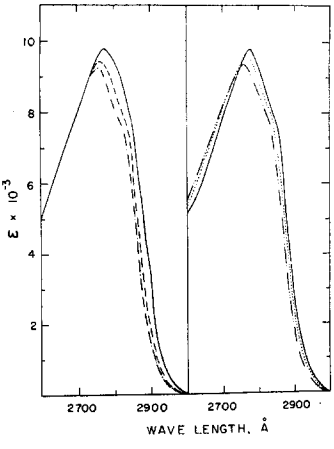

Fig. 1. Absorption spectra of: ——— ribonuclease in water, or in phosphate buffer, or in a solution 8 M in urea and 0.15 M in phosphate buffer pH 6.5; ----- PIR in water; —·—· ribonuclease, or PIR, after digestion with pepsin at pH 1.8, read at pH 6.5; —···—··· ribonuclease in 8 M urea; ····· ribonuclease in a solution 8 M in urea and 0.003 M in phosphate buffer pH 6.5.

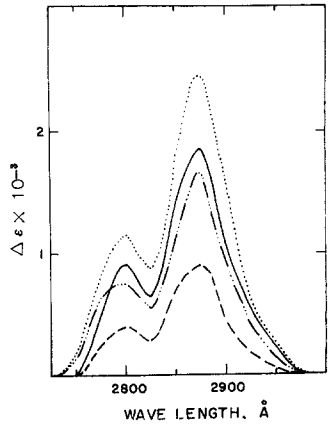

Fig. 2. Decrease in molar extinction at pH 6.5, relative to the spectrum of native RNase, of: ——— PIR; ····· final peptic digest of RNase or PIR; —···—··· RNase in 8 M urea; ----- RNase in 8 M urea and 0.003 M phosphate buffer.

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Fig. 2. Difference spectra calculated from the data of SHUGAR[15], show similar maxima and minima (see also SCHERAGA[16]). The change in molar extinction at 2850 A has been plotted as a function of time in Fig. 3 and, for comparison, the ribonuclease activity of the digestion mixture has been super-imposed after normalizing the data on a percentage basis. The data in Fig. 3 indicate the complete correspondence of the kinetics of spectral change with activity change, and, thus, on the basis of results published previously[7], with the appearance of the free tetrapeptide released during pepsin inactivation. It must be emphasized that the absorption data in Fig. 3 represent the summation of effects contributed by RNase, by PIR, as well as by subsequent non-specific products of further proteolysis. Thus it can be shown, by examination of the spectrum of purified PIR before and after further pepsin treatment, that an additional shift in the curve occurs during subsequent proteolysis (see Fig. 1)*.

Curves for both native RNase and extensive pepsin digests show still a further shift at pH 1.8 as compared with the same solutions at neutral pH values[15]. This observation, which has been further studied by SCHERAGA[16] and ourselves, emphasizes

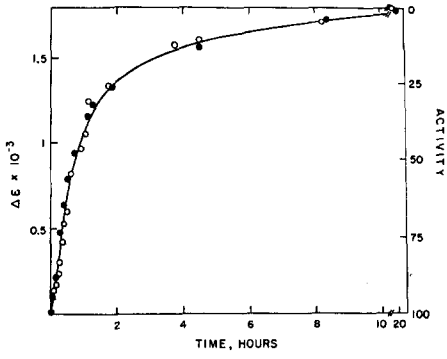

Fig. 3

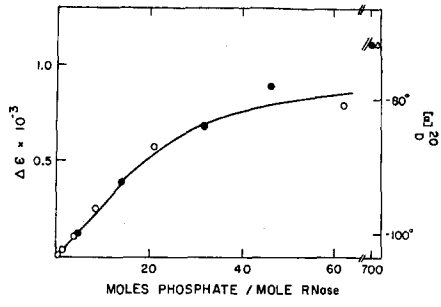

Fig. 4

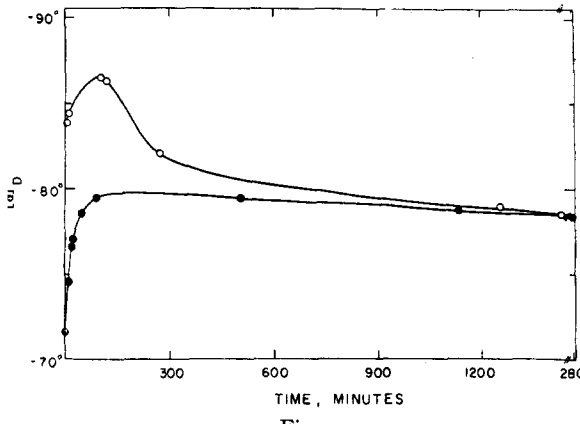

Fig. 5

Fig. 3. Decrease in absorption (measured at 2850 A, pH 6.5) and in activity (expressed as percentage of native RNase) of RNase upon peptic digestion (c_{RNase} = 2 g/l; c_{pepsin} = 0.0025 g/l; pH 1.8; 25° C). —●— decrease in molar extinction; —○— activity.

Fig. 4. Influence of phosphate ions on the molar extinction at 2850 A, and optical rotation, of RNase in 8 *M* urea solution. —●— increase in molar extinction, at pH 6.5, relative to the spectrum of RNase in 8 *M* urea; —○— optical rotation, at pH 6.5; —△— this point corresponds to the molar extinction and optical rotation of RNase in water in the absence of salt and urea. The spectrophotometric readings were carried out on RNase solutions containing 2.45 g/l; polarimetric readings — on solutions of 27 g/l.

Fig. 5. The optical rotation of: —●— native RNase (c = 30 g/l) treated with pepsin (c = 0.035 g/l); and —○— PIR (c = 11 g/l) treated with pepsin (c = 0.05 g/l). The reactions were carried out at pH 1.8 and 25°.

* Shift in U.V. absorption upon pepsin digestion of serum globulin and albumin has been reported by BEAVEN AND HOLIDAY[17].

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the multiplicity of the changes in tertiary structure which may lead to shifts in absorption characteristics. As will be discussed below, these changes can, by no means, be identical. Following the shift that occurs under acid conditions, for example, enzymic activity is unimpaired, whereas the shift produced by limited pepsin digestion parallels inactivation.

Spectral change in urea

HARRINGTON AND SCHELLMAN[18] have shown that a shift in spectrum, quantitatively similar to that occurring during pepsin digestion, occurs when ribonuclease is dissolved in 8 *M* urea solutions over a wide pH range. This shift, accompanied by no change in catalytic activity, has now been studied with respect to the ionic environment of the protein. In all cases RNase, urea, and salt, were dissolved at the same time, and the readings were carried out at least 15 minutes after solution. No change in readings occurred after 24 hours. The data in Table I show the complete prevention of spectral change by phosphate and arsenate ions, a partial prevention by sulfate ions, and essentially no effect by chloride, bromide, and acetate ions. The data in Fig. 4 show that the shift in spectrum produced by urea is strongly inhibited (50%) by levels of phosphate (22/mole) approaching the number of basic groups in the protein (19/mole). The shift induced by limited pepsin digestion is, on the other hand, unaffected by phosphate ions, an observation which further emphasizes the basic differences between the superficially similar tertiary structural changes induced by different experimental treatments.

TABLE I

THE OPTICAL ROTATION AND MOLAR EXTINCTION, AT 2850 A, OF RNASE IN 8 *M* UREA IN THE PRESENCE OF VARIOUS ANIONS (AS SODIUM SALTS)*

Anion	*Molarity*	*Urea molarity*	$\Delta\varepsilon \times 10^{-3}$**	$[\alpha]_D^{20}$
Salt-free	—	8	0	—103.7°
Acetate	0.50	8	0	—
Chloride	0.40	8	110	— 98.8°
Bromide	0.50	8	150	—
Sulfate	0.05	8	—	—102.5°
Sulfate	0.25	8	840	— 88.0°
Arsenate	0.15	8	1120	— 80.7°
Pyrophosphate	0.15	8	1120	—
Phosphate	0.001	8	112	—103.7°
Phosphate	0.003	8	390	—102.9°
Phosphate	0.007	8	670	—
Phosphate	0.01	8	900	— 99.9°
Phosphate	0.02	8	—	— 96.5°
Phosphate	0.05	8	—	— 87.0°
Phosphate	0.15	8	1120	— 81.0°
Salt-free	—	—	1120	— 71.7°

* c_{RNase} = 2.45 g/l for spectrophotometric readings, and 27 g/l for polarimetric readings.
** Increase relative to spectrum of RNase in 8 *M* urea in the absence of salts.

The optical rotation of ribonuclease in urea

The inhibition of the change in spectral properties of ribonuclease by multivalent anions suggested the further study of structural modification by examination of

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optical rotatory characteristics. HARRINGTON AND SCHELLMAN[18] have shown that the optical rotation of RNase is markedly changed in 8 *M* urea, and in a direction concordant with the disorganization of tertiary structure (see also YANG AND DOTY[19]). Table I contains in addition to the $[\alpha]_D^{20}$ values found for native and urea-denatured RNase, data indicating the effect of various anions on rotatory changes caused by urea. Although the inhibition of the shift in optical rotation is not absolute, it can be seen (Fig. 4) that phosphate ions do, indeed, almost completely inhibit such a shift and at a mole ratio of anion to protein very similar to that found in the case of the spectral studies discussed above.

SIMPSON AND KAUZMANN[20] have shown that the optical rotatory changes occurring during urea denaturation of ovalbumin are similarly influenced by anions. Their experiments demonstrated that the change in the final rotation of ovalbumin solutions in urea was a linear function of salt concentration and was of the order of 25° mole^{-1} liter for univalent, and 50° mole^{-1} liter for multivalent anions. The present effects of phosphate on the ribonuclease-urea system are of the order of 410° mole^{-1} liter calculated from the initial slope, indicating a higher order of interaction specificity*. The inhibition of denaturation of various proteins by salts, as measured by several criteria, has been reported in the literature (*e.g.*, BURK[21]), but an integrated discussion of this subject is clearly not feasible in the present communication.

Optical rotation of PIR

It is now reasonably well established that the structural "unfolding" of proteins (brought about, for example, by hydrogen-bond rupturing agents such as urea[18]) is reflected in an increase in the negative optical rotation[22,19]. Extensive proteolysis causes additional changes in the optical rotation[23]. If, as previous physical and chemical information indicates, PIR represents a derivative of RNase in which only a minor structural change has occurred, this material should exhibit optical rotatory properties quite similar to the native enzyme. The data summarized in Fig. 5 show that the $[\alpha]_D^{20}$ of PIR (—83.8°) is only slightly more negative than that for RNase, and further emphasize the relatively minor change in structure which occurs in going from the native enzyme to PIR.

The optical rotatory changes occurring during extensive pepsin digestion of both PIR and of native RNase are also summarized in Fig. 5. Of particular significance is the fact that the final rotations reached for both materials are identical. An attempt to rationalize the shapes of the kinetic curves in terms of unfolding and proteolysis would be sheer speculation on the basis of the limited data on hand.

The influences of alkali, ammonia and heat

SHUGAR[15] and TANFORD *et al.*[24] have concluded from spectrophotometric experiments that three of the six tyrosine residues in ribonuclease titrate anomalously, ionizing only at pH values about 12.5, and in a manner leading to hysteresis upon reverse titration. Upon examination of ribonuclease solutions kept for two hours at room temperature at various alkaline pH values (NaOH) and afterwards neutralized (HCl),

* A. M. CRESTFIELD AND F. W. ALLEN, *J. Biol. Chem.*, 211 (1954) 363, have assumed an extensive binding of phosphate ions by ribonuclease to explain the decrease in the isoelectric pH as a function of phosphate ion concentration.

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we have found that RNase suffers irreversible activity loss, in a fashion which parallels spectral change, at pH values higher than around 12.7, where 40% loss occurs in two hours. The enzyme activity and spectrum are, however, completely unchanged at lower pH values as well as following solution of RNase under the same conditions both in liquid ammonia and in 28% aqueous ammonia. At 100° C, slow loss of activity occurs (35% loss in 2 h) and the rate of loss is accompanied by a spectral shift.

CONCLUSIONS

At least four distinct phenomena which might involve changes in hydrogen bonding appear to be demonstrable by a study of the spectrophotometric and rotatory properties of RNase. In the case of exposure to *low pH values* the ultraviolet shift might also be due to inductive effects brought about by the acquisition of protons by carboxylate ions vicinal to tyrosine residues in the protein chain. *In alkali*, TANFORD *et al.*[24,25] have suggested that the modifications involve changes in "hydrophobic" centers superimposed on hydrogen bond rupture. *Urea-induced changes* would appear to follow massive disorientation of the structure. Finally, *limited pepsin digestion* appears to exert its effect through a rather local modification of RNase.

It is of interest to contrast the reversible structural effects brought about without loss of activity by urea and by acid, with the irreversible effects of high pH and of pepsin digestion on activity and physical properties. Two immediate possibilities suggest themselves as explanations for this dichotomy. First, the same three anomalous tyrosine residues may be involved in the ultraviolet shifts observed under all four experimental treatments and the loss in activity may be related to changes in one or more structural configurations which are not related directly to the shift. Second, the shifts, although in some cases numerically similar, may be due, for example, to the modification of tyrosines A and B by an agent not affecting activity, and B and C by an agent which does. A rational picture of these changes in tertiary structure will depend on the progress in our knowledge of the sequence[26,27] and the location of disulfide bridges[28] of RNase and of the changes brought about by controlled degradative modifications of the native molecule.

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SUMMARY

Evidence is presented supporting the conclusion that limited pepsin hydrolysis of native bovine ribonuclease results in the accumulation of a derivative which differs, covalently, from the native enzyme only in lacking the C-terminal tetrapeptide. Spectrophotometric and polarimetric studies of this derivative, and of native RNase, exposed to urea, low and high pH values, and heat, suggest that a variety of changes in the tertiary structure may occur, some of which lead to inactivation while others do not.

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X-RAY MICRORADIOGRAPHY OF TISSUE SECTIONS WITH MAGNESIUM RADIATION

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In a recent note[1] we showed that by using aluminum foil as target and by circulating helium in the enclosed specimen and photographic chamber, projection X-ray microradiographs giving good contrast could be made from sections of soft tissue. We have now found that still better contrast, sufficient to reveal even more structural detail, can be obtained by employing magnesium foil as target and evacuating the space between target and photographic plate. Though the two elements are adjacent in the periodic table, the K radiation of magnesium, with a wavelength of *ca.* 10 A, has an absorption considerably higher than that of aluminum. Furthermore, the low

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