The inactivation of ribonuclease by restricted pepsin digestion

Recent studies on the chemical and physical properties of ribonuclease[1-6] begin to permit a rational examination of the structural basis of its enzymic activity. Ribonuclease consists of a single peptide chain[1] of 126 amino acids folded into a relatively compact structure under the influence of four disulfide cross-linkages and a secondary network of non-covalent bonds. The former may be broken by controlled oxidation yielding a molecule without enzymic activity[1]. The latter are easily ruptured in strong urea or guanidine chloride solutions and appear to be nonessential from the standpoint of catalytic activity since the urea "denatured" molecule, possessing a degree of disorientation nearly as great as the randomly coiled oxidized chain, retains the catalytic function of the native enzyme[5].

The native ribonuclease molecule has been subjected to the limited action of several proteolytic enzymes and, on the basis of the structural studies mentioned above, the sites of attack of these proteases can be considered in relation to the enzymically active region of the molecule. Thus, the extreme C-terminal end of the chain does not appear to be catalytically involved since several amino acid residues may be removed by carboxypeptidase without loss in activity[7]. Similarly, limited attack by the bacterial protease, subtilisin, yields a fully active derivative containing a new N-terminal end group, the fairly short chain which it terminates being apparently attached to the body of the molecule by disulfide bonding[8,9].

Earlier studies on the action of pepsin on ribonuclease suggested that an initial product was produced differing only very slightly from native ribonuclease[10,11]. Its S^{20}_W and α-amino nitrogen content were, within experimental error, unchanged in spite of complete loss of enzymic activity. This reaction has now been studied in greater detail and its kinetics have been examined by the chromatographic procedure of HIRS, MOORE AND STEIN[12]. The isolation and partial characterization of the initial product suggest that a very limited change has occurred within the enzymically active portion of the molecule.

In Fig. 1 are shown the behaviour of solutions of ribonuclease and ribonuclease digests on IRC-50 columns following digestion with pepsin at pH 1.8 for short periods of time. In these experiments, the action of pepsin was stopped by adjustment of the pH to 6.5. The figure shows the progressive production of a new chromatographic species at the expense of the native enzyme (appearing in the usual double peak form, both peaks being fully active). As shown, the new derivative is devoid of catalytic activity against RNA as tested by measurement of the production of uranylacetate soluble fragments[1]. Chromatography of pepsin-treated ribonuclease at pH 6.05 rather than 6.45 as employed here does not permit the elution of the inactive molecule from IRC-50 columns even after long periods of development. By paper chromatography (in butanol-acetic acid-water; 4:1:5) of samples of the digestion mixture taken after different time periods, it was found that a peptide was released in progressively larger amounts. This peptide contained aspartic acid, serine, alanine and valine. The rate of appearance of the fragment was qualitatively correlated with the rate of production of the inactive protein component in Fig. 1.

To characterize the new protein derivative more fully, large samples of digest (identical with the smaller sample employed for chromatogram C in Fig. 1 were run through large columns under the same conditions as those described above. The flow rate was adjusted to a speed which permitted the separation of the main inactive component from the more drastically changed products, shown in Fig. 1-C as a shoulder on the principal inactive peak. The purified derivative was freed of salt by dialysis against distilled water and lyophilized, as was the remaining native enzyme which had emerged in earlier tubes.

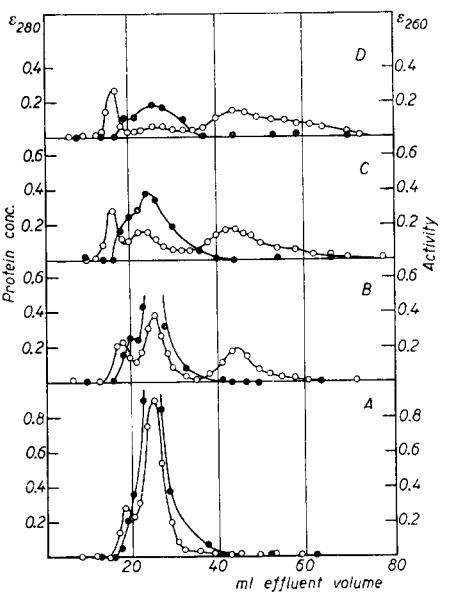

Fig. 1. Chromatography (12) of 10 mg samples of native and pepsin-digested ribonuclease on IRC-50 columns, 9 × 30 mm. Eluting buffer, phosphate, 0.2 *M*, pH 6.45. Digestion conditions: ribonuclease 0.92%; pepsin (Armour crystallized) *ca.* 0.002%; 37° C, pH 1.8. Remaining ribonuclease activity (1): Curve A, 100%; B, 60% (5 min); C, 40% (10 min); D, 15% (16 min). Solid points, enzyme activity. Open circles, protein concentration. (The peak appearing at 18–19 effluent ml is a ninhydrin-negative artefact, uniformly seen with this preparation of resin.)

Both the active and inactive components showed the same sedimentation constant, S^{20}_W, (1.8–1.9 for 1% solutions) and intrinsic viscosity (3.3–3.9) indicating that the physical shape and size had not been significantly altered. Performic acid oxidation of the two materials yielded products with comparable sedimentation characteristics (S^{20}_W (infinite dilution) = 1.78) although the sedimentation constant of the oxidized pepsin derivative showed somewhat less concentration dependence (S^{20}_W (1%) for oxidized native RNase, 1.43; for oxidized derivative, 1.60). The reduced viscosity (1%) of the oxidized derivative (10.3) was slightly less than that of the oxidized native enzyme (12.3).

Upon filter paper electrophoresis of the oxidized compounds at pH 8.6 in barbital buffer the inactive material was separated from the native enzyme, being more electropositive. The oxidized pepsin-modified molecule showed a second, neutral, component which was less reactive to ninhydrin. The characterization of this fragment which was presumable linked to the body of the inactive molecule through disulfide linkage is now in progress.

The present studies clearly exclude the occurence of the "all-or-none" or "explosive" proteolysis suggested in earlier work with pepsin and various protein substrates at pH values in the neighborhood of 1.8–2.0[14,15,16,17]. They support the general mechanism of stepwise proteolysis indicated by investigations of the action of subtilisin and other proteases[8,9,18,19] on protein substrates and reinforce the probability of the generality of such a mechanism.

The inactivation of ribonuclease following a minimal change in its fine structure by pepsin is of particular interest in conjunction with the earlier findings on urea unfolding and on the subtilisin digestion of ribonuclease. Together with these observations, the present data support the view that the enzymically active center of ribonuclease may be dependent on only a relatively small portion of the protein structure and suggest that the complexities of macromolecular structure may be associated with secondary non-catalytic phenomena perhaps related to cellular architecture.

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