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Immobilized Carboxypeptidase A as a Probe for Studying the Thermally Induced Unfolding of Bovine Pancreatic Ribonuclease†

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ABSTRACT: A method for the preparation of Sephadex-immobilized carboxypeptidase A is presented. This form of the enzyme has the same specific activity as the soluble enzyme at room temperature, but retains its activity at higher temperatures (60–70°). This preparation of immobilized carboxypeptidase A was used, as a proteolytic probe, to investigate the thermally induced unfolding of the C-terminus of ribonuclease A. This technique indicates that the C-terminal residues of ribonuclease A do not unfold until the high-temperature region of the thermal transition (as determined by ultraviolet difference spectrophotometry and optical rotation).

While a considerable amount of information is available about the three-dimensional structures of many proteins, there is little knowledge about the pathway(s) by which the nascent polypeptide chain attains its unique native conformation. For a protein, like bovine pancreatic ribonuclease, which contains disulfide bonds, we do not even know whether the pathway(s) deduced for the folding of the reduced protein (Hantgen *et al.*, 1974) is (are) the same as that (those) for the folding of the denatured protein with its disulfide bonds intact. It is therefore desirable to obtain experimental evidence relevant to the pathway(s) of protein folding and unfolding. One technique sometimes used to study the stages of protein unfolding involves the use of proteolytic enzymes; it is assumed that portions of the polypeptide chain which unfold become accessible to the proteolytic enzyme (Mihalyi and Harrington, 1959; Rupley and Scheraga, 1963; Ooi *et al.*, 1963; Scott and Scheraga, 1963; Ooi and Scheraga, 1964; Klee, 1967). Some of the aforementioned experiments provided information about the sequence in which certain peptide bonds become accessible to proteolytic enzymes, as the temperature is raised in the region of the thermal transition of bovine pancreatic ribonuclease. In this paper, we present some new experimental evidence, obtained by proteolytic digestion with immobilized carboxypeptidase A, which indicates that the C-terminal portion of ribonuclease unfolds at the high-temperature end of the thermal transition. These experiments were carried out on *equilibrium* systems at various stages of the thermally induced unfolding of ribonuclease.

Unfortunately, it was difficult to draw quantitative conclusions from many of the earlier proteolytic digestions of ribonuclease at elevated temperatures because of (i) the unfolding of the proteolytic enzyme and (ii) subsequent autolysis. In this paper, immobilized carboxypeptidase A was used as the proteolytic probe, and as a consequence it has been possible to obtain quantitative results. The susceptibility of ribonuclease to digestion by carboxypeptidase A was reported previously (Klee, 1967). In that work, soluble carboxypeptidase A was used, and above 55° it appears that the denaturation of the proteolytic probe was too rapid to yield accurate results. Klee (1967) reported that the rate of digestion of performic acid oxidized ribonuclease A was significantly less than the rate of digestion of thermally denatured ribonuclease A at 57.5°. This result is difficult to understand, and was one of the reasons that we attempted to apply this approach again using a more stable probe, *viz.*, immobilized carboxypeptidase A.

Materials and Methods

Bovine pancreatic ribonuclease A (lot 3cc) and diisopropyl fluorophosphate treated carboxypeptidase A (lot 6125) were obtained from Worthington Biochemical Corporation. *N*-Cbz-glycyl-L-phenylalanine (lot F3916) was obtained from Mann Research Laboratories. All other reagents were analytical grade, and deionized, distilled water was used throughout this work.

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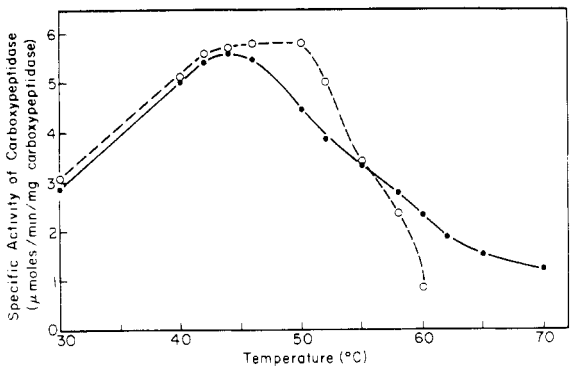

FIGURE 1: The rate of hydrolysis of *N*-Cbz-Gly-L-Phe by carboxypeptidase A [(○) soluble enzyme; (●) immobilized enzyme] as a function of temperature at pH 6.83, ionic strength, 0.16.

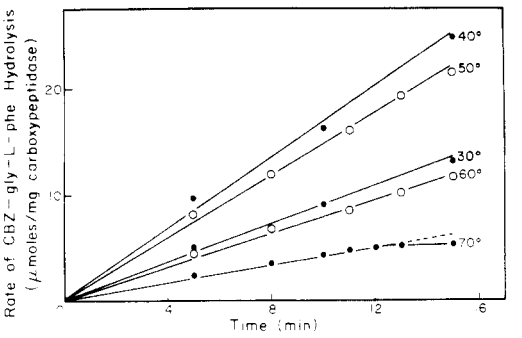

FIGURE 2: The time dependence of the rate of hydrolysis of Cbz-Gly-L-Phe by immobilized carboxypeptidase A at different temperatures (pH 6.83, ionic strength 0.16).

The preparation of immobilized carboxypeptidase A was carried out by a procedure similar to that of Axén and Ernback (1971). Sephadex G-200® was used as a support, since agarose shows conformational transitions around 50° (Gabel and Kasche, 1973) which might have interfered with the studies reported here. Activation of Sephadex G-200 with cyanogen bromide at pH values above 10.0, with uncontrolled reaction (*i.e.*, addition of solid CNBr), and with prolonged reaction times, leads to considerable cross-linking of the gel with concomitant decrease in bed volume (Axén and Vretblad, 1971) and reduced activity of the enzyme against high-molecular weight substrates (Axén and Ernback, 1971); 200 mg of Sephadex G-200 was suspended in 5 ml of water; 200 mg of cyanogen bromide, dissolved in 4 ml of water, was added, and the activation was started by raising the pH to 10.0 and maintaining it there for 6 min by means of a pH-Stat. The activated gel was washed on a glass filter with 200 ml of cold water and 100 ml of the buffer used for coupling (0.1 M $NaHCO_3$ (pH 8.2)); 25 mg of diisopropyl fluorophosphate treated carboxypeptidase A was dissolved in 5 ml of the same buffer and added to the activated gel. Coupling was allowed to proceed overnight in the cold by tumbling the coupling vessel end-over-end. The gel was inserted into a small column, and the protein that was not covalently bound to the carrier was removed by washing at room temperature with the following buffers (flow rate about 10 ml/hr): (1) 0.1 M $NaBO_3$–0.5 M NaCl (pH 8.0, 8 hr); (2) 0.1 M NaOAc–0.5 M NaCl (pH 5.0, 24 hr); (3) 0.1 M $NaBO_3$–0.5 M NaCl–0.2 M glycine (pH 8.0, 24 hr); (4) 0.1 M $NaBO_3$–0.5 M NaCl (pH 8.0, 24 hr); (5) 0.1 M NaOAc–0.5 M NaCl (pH 5.0, 24 hr); (6) 0.16 M KCl (pH 6.83, 10 hr). All of these buffers contained 1 mM $ZnCl_2$ to prevent the loss of Zn^{2+} from the active site of carboxypeptidase. When no $ZnCl_2$ was added to the washing buffers, the immobilized carboxypeptidase lost activity. The amount of protein covalently bound in the conjugate was determined by amino acid analysis after acid hydrolysis; 18% of the added carboxypeptidase A was found to be coupled to the activated carrier; there was 22.4 mg of carboxypeptidase per gram of conjugate. The immobilized enzyme was stored as a suspension in the final washing buffer at 4°, and showed no decrease in activity after 9 months.

The activity of immobilized carboxypeptidase A toward *N*-Cbz-Gly-L-Phe was measured in a thermostated chamber under nitrogen. The temperature of the reaction vessel was maintained (±0.2°) using a Haake circulating water bath. A Radiometer Model TTT1a pH-Stat, equipped with a scale expander (accurate to 0.01 pH unit), was used to maintain the reaction solution at pH 6.83 ± 0.05. The substrate (0.25–2.0 mM in 0.16 M KCl–1 mM $ZnCl_2$) was equilibrated at the desired temperature for at least 5 min, at which time immobilized enzyme (equilibrated to the same temperature for 1 min) was added. The rate of hydrolysis was measured by following the release of phenylalanine at 5, 10, and 15 min for all temperatures (except 70° where the reaction rate was constant only for 12 min). To determine the concentration of phenylalanine released at any time, the reaction was terminated by the addition of 1 ml of 0.1 M HCl. The immobilized carboxypeptidase was removed by centrifugation, and the decrease in absorbance at 223.5 nm (Hofmann and Bergman, 1940; Neurath *et al.*, 1947) was measured using a Zeiss Model PMQ-II ultraviolet spectrophotometer. The above procedure was also used to assay for soluble carboxypeptidase A, except that the proteolytic enzyme was not removed; at the higher temperatures (greater than 60°) difficulty was encountered because of the rapid denaturation of the soluble enzyme (at 70° a constant rate of hydrolysis could be maintained only for 0.5 min).

Performic acid oxidized, and also unoxidized, ribonuclease A were hydrolyzed by immobilized carboxypeptidase A at various temperatures. The performic acid oxidized ribonuclease was prepared by the procedure described by Hirs (1956); 30 mg of oxidized ribonuclease A (in 3 ml of 0.16 M KCl–1 mM $ZnCl_2$ (pH 6.83)) was equilibrated at a given temperature and subjected to proteolysis by 1 mg of immobilized carboxypeptidase A-Sephadex conjugate for 10 min. The reaction was terminated using 1 ml of a 50% solution of trichloroacetic acid. The precipitate was removed by a bench centrifuge, and the supernatant fluid was used for amino acid analysis. A Beckman Model 120 automatic amino acid analyzer, equipped to detect 3–25 nmol of amino acid, was used to determine the amount of free amino acids in solution. The buffer system used for the analysis was (1) 0.20 N sodium citrate (pH 3.25) for 50 min, (2) 0.35 N sodium citrate (pH 5.25) for 70 min. The same procedure was followed in the analysis of the reaction of immobilized carboxypeptidase with unoxidized ribonuclease A at different temperatures.

Results and Discussion

The hydrolysis of ribonuclease by proteases is difficult to measure accurately as a function of temperature because the proteolytic enzymes also undergo a thermal transition, which results both in autolysis and loss of activity. This is particularly evident in the earlier studies with α-chymotrypsin (Rupley and Scheraga, 1963), trypsin (Ooi *et al.*, 1963) and carboxypeptidase A (Klee, 1967). The problem has been solved partly, here, by immobilizing the carboxypepti-

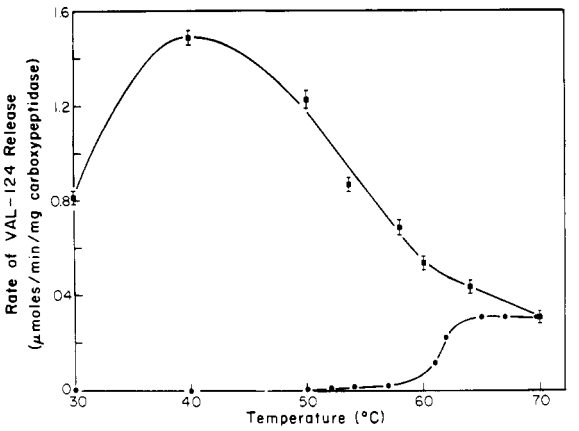

FIGURE 3: Rate of release of Val-124 from performic acid oxidized ribonuclease A (■) and unoxidized ribonuclease (●) by immobilized carboxypeptidase A at different temperatures (pH 6.83, ionic strength, 0.16).

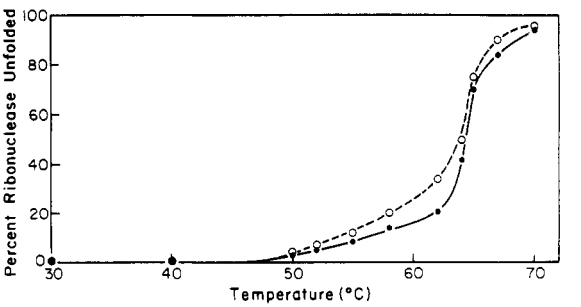

FIGURE 4: A comparison of the percentage unfolding of ribonuclease A as measured by ultraviolet difference spectrophotometry and optical rotation (○) (Hermans and Scheraga, 1961) and by hydrolysis by immobilized carboxypeptidase A (●) at different temperatures.

dase A on Sephadex G-200. The initial rate of hydrolysis of the peptide *N*-Cbz-Gly-L-Phe by carboxypeptidase A in solution and by immobilized carboxypeptidase A at pH 6.8 and ionic strength 0.16 is given in Figure 1. Although the specific activity of the soluble carboxypeptidase A is greater than that of the immobilized enzyme near 50°, upon reaching 60° the activity of the immobilized enzyme is nearly three times that of the soluble enzyme. Indeed, above 60° (at pH 6.83) the rate of denaturation of the soluble carboxypeptidase is so large that it cannot be used to study the thermal unfolding of ribonuclease A. Examination of the results from a previous study of the action of soluble carboxypeptidase A on ribonuclease as a function of temperature emphasizes the difficulties in obtaining accurate results above 50° (Klee, 1967). The maximum rate of hydrolysis of *N*-Cbz-Gly-L-Phe occurred at 44° for the immobilized carboxypeptidase, but the enzymatic activity of this preparation persisted for much longer (and at higher temperature) than that of the soluble enzyme. The activity of immobilized carboxypeptidase A was sufficiently stable to obtain a linear rate of hydrolysis of Cbz-Gly-L-Phe for over 10 min (see Figure 2). Although the rate of hydrolysis is lower at 70° (compared to 50°), the equilibrated immobilized carboxypeptidase A does not lose activity during the measurement; *i.e.*, there is no further denaturation of immobilized carboxypeptidase over the time course of the experiment.

In following the action of carboxypeptidase A on ribonuclease, there was no observed liberation of Ser-123 during the time that liberation of Val-124 was measured. The temperature dependence of the initial rate of hydrolysis of oxidized ribonuclease A and unoxidized ribonuclease A by immobilized carboxypeptidase A is shown in Figure 3. A comparison of Figures 1 and 3 shows that the temperature dependence of the hydrolysis of oxidized ribonuclease is the same as that of the hydrolysis of Cbz-Gly-L-Phe. This indicates (i) that any changes in the characteristics of oxidized ribonuclease as a function of temperature do not affect the penetration of the chain into the conjugate, and (ii) the structural changes at the catalytic site with temperature are the same for both substrates. A similar experiment using the ester hippuryl-L-phenyllactic acid showed different temperature characteristics, the optimum temperature being ~59°. The difference in temperature maxima suggests that a different part of the catalytic site is involved during ester hydrolysis; presumably this part of the site changes structure at a higher temperature than the site involved in peptide hydrolysis. A comparison of the rates shown in Figure 1 for Cbz-Gly-L-Phe and in Figure 3 for the ribonucleases shows that the immobilized carboxypeptidase A hydrolyzes the model substrate four times faster than the polypeptide. Since all substrates are at similar molar concentrations, the difference in rate presumably reflects both the relative affinities of carboxypeptidase for the phenylalanine and valine side chains, respectively, and the partial steric exclusion of the polypeptide chain from the active site of the immobilized carboxypeptidase A (Axén and Vretblad, 1971). As distinct from the earlier results on the action of carboxypeptidase on ribonuclease (Klee, 1967), the results shown in Figure 3 indicate that thermally denatured ribonuclease and oxidized ribonuclease are hydrolyzed at the same rate by carboxypeptidase A; *i.e.*, the hydrolysis rates at 70° are identical for the two ribonucleases. Any residual structure remaining in the thermally denatured ribonuclease does not affect the rate of hydrolysis by carboxypeptidase, nor does the increased charge of oxidized ribonuclease affect the rate of hydrolysis significantly.

At each temperature, the rate of hydrolysis of oxidized ribonuclease was assumed to be the same as that of thermally unfolded ribonuclease. This assumption is justified by the coincidence of the two curves of Figure 3 at 70°. Using the data of Figure 3, the per cent of unfolded protein at each temperature was then calculated as

$$\%\ \text{unf. at temp. T} = \frac{\text{rate of hydrolysis of partially denatured ribonuclease}}{\text{rate of hydrolysis of oxidized ribonuclease}} \times 100\%$$

assuming that oxidized ribonuclease A is 100% unfolded (compare with Klee, 1967).

The percentage unfolding of ribonuclease in these experiments actually reflects the accessibility of the C-terminal amino acid to the exopeptidase. In Figure 4, the results are compared directly with those from ultraviolet difference spectrophotometry and optical rotation (under the same conditions) reported by Hermans and Scheraga (1961); the spectrophotometric data reflect the degree of exposure of tyrosine residues at each temperature. The unfolding of the C terminus is not as great as the change in exposure of the tyrosine residues between 50 and 61°. After the transition temperature (62°, by ultraviolet difference spectrophotometry), the C-terminus unfolds to the same extent as the tyrosine exposure. This suggests that at least some of the perturbed tyrosine residues are exposed before any definite changes occur in the C-terminal region of ribonuclease. Once the C-terminus starts to unfold, however, the rate of

exposure of new tyrosines parallels the rate of exposure of the C-terminus. This observation is consistent with the notion that tyrosine residues 92 and 25 unfold (Bigelow, 1961; Scott and Scheraga, 1963; Li *et al.*, 1966) before the C-terminal region "melts." In the temperature range in which the C-terminal region unfolds, Tyr-97 becomes exposed to solvent (Li *et al.*, 1966), although the hydroxyl group must remain in an ordered region of the polypeptide because the spectrum of this residue is normalized only in 8 M urea (Bigelow, 1961).

In summary, the C-terminal region of ribonuclease unfolds at higher temperatures than some of the abnormal tyrosine residues begin to normalize, in the reversible thermal denaturation of this protein.

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Added in Proof

In a related paper (A. W. Burgess and H. A. Scheraga, *J. Theor. Biol.*, submitted), a mechanism has been presented for the pathway of unfolding of ribonuclease. In that mechanism, the C-terminal residues were postulated to unfold late in the thermal transition. The present paper constitutes experimental evidence for that aspect of the mechanism.

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Identification of the Lysine Residue Modified during the Activation by Acetimidylation of Horse Liver Alcohol Dehydrogenase†

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ABSTRACT: A single amino group in horse liver alcohol dehydrogenase was modified with methyl [^{14}C]acetimidate by a differential labeling procedure. Lysine residues outside the active site were modified with ethyl acetimidate while a lysine residue in the active site was protected by the formation of an enzyme-NAD^+-pyrazole complex. After the protecting reagents were removed, the enzyme was treated with methyl [^{14}C]acetimidate. Enzyme activity was enhanced 13-fold as 1.1 [^{14}C]acetimidyl group was incorporated per active site. A labeled peptide was isolated from a tryptic-chymotryptic digest of the modified enzyme in 35% overall yield. Amino acid composition and sequential Edman degradations identified the peptide as residues 219-229; lysine residue 228 was modified with the radioactive acetimidyl group.

Jörnvall (1973) and Tsai *et al.* (1974) have suggested that reductive methylation of several lysine residues of horse liver alcohol dehydrogenase (EC 1.1.1.1) increases the enzymatic activity, while Plapp *et al.* (1973) showed that amidination of a single residue increases the turnover numbers tenfold. Steady-state and stopped-flow kinetic studies show that the enchanced activity is due to increases in the rate-limiting steps of the forward and reverse reactions, namely the dissociation of the enzyme-coenzyme complexes (Plapp, 1970; Plapp *et al.*, 1973). In order to understand the role of the amino group(s) in the activity of the enzyme we must locate the modified lysine residue(s) in the primary and tertiary structure. In the present work, one lysine residue per active site was differentially amidinated with methyl [^{14}C]acetimidate and identified.

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