Structural Studies of Ribonuclease. XVIII. An Investigation of the Peptic Digestion Products of Ribonuclease*

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ABSTRACT: The peptic digestion of ribonuclease A at pH 2.0 (dilute HCl) and at 25° was extensively studied. The intermediates have been fractionated by column chromatography, and the sites of peptic cleavage have been identified as Phe-Asp (120-121), Met-Ser (79-80), Thr-Phe (45-46), Glu(NH_2)-Ala (55-56), and Phe-Glu (8-9). Since all the high molecular weight intermediates studied were missing the C-terminal tetrapeptide and had no enzymic activity, it seems reasonable that the unfolding of the C terminus of ribonuclease A at low pH is essential for the peptic cleavage of the Phe-Asp bond. Cleavage of the other bonds may arise either from this unfolding, or from a subsequent unfolding which results from the removal of the C-terminal tetrapeptide. One of the large intermediates has been shown to be identical with Anfinsen's pepsin-inactivated ribonuclease (PIR) [Anfinsen, C. B. (1956), *J. Biol. Chem. 221*, 405], on the basis of both chemical analyses and spectrophotometric titration experiments. Thus, PIR has been shown to be produced under the milder conditions of peptic hydrolysis used here, just as under Anfinsen's conditions (pH 1.8, 37°), in contrast to the results of A. Ginsburg and H. K. Schachman [(1960), *J. Biol. Chem. 235*, 115] which suggested the formation of an active intermediate under the similar milder conditions used in the present work.

One or more of the three abnormal tyrosyl residues of ribonuclease (Shugar, 1952; Tanford *et al.*, 1955) probably interact with carboxyl groups and are embedded in a fairly nonpolar environment (Hermans and Scheraga, 1961a,b). Two of the abnormal tyrosyl residues and the three abnormal carboxyl residues have been identified (Cha and Scheraga, 1963; Donovan, 1963; Riehm *et al.*, 1965). However, the problem of identifying which carboxyl group reacts with each tyrosyl group remains. This question may be approached by studying pepsin-inactivated ribonuclease (PIR)[1] (Anfinsen, 1956), which has only one abnormal tyrosyl residue (Bigelow and Ottesen, 1959). We *assume* that the abnormal tyrosyl residue of PIR is one of the three abnormal residues of ribonuclease (Bigelow, 1961), and that the other two tyrosyl residues have been normalized because of the looser structure of PIR (Sela and Anfinsen, 1957; Sela *et al.*, 1957; Ottesen and Stracher, 1960). We, therefore, seek to identify the one abnormal tyrosyl residue of PIR.

Anfinsen (1956) showed that PIR is obtained as the first digestion product when pepsin removes the C-terminal tetrapeptide (Asp-Ala-Ser-Val) of ribonuclease; he also showed that PIR is enzymically inactive, and that the loss of activity paralleled the release of the tetrapeptide. Anfinsen carried out the digestion at pH 1.8 and 37°, conditions under which ribonuclease is partially unfolded (Hermans and Scheraga, 1961a). On the other hand, Ginsburg and Schachman (1960) reported the existence of an enzymically active product obtained by peptic digestion under milder conditions (pH 2.1–2.2 at 22–26°), where ribonuclease is only slightly unfolded (Hermans and Scheraga, 1961a).

The purpose of this study was, therefore, to characterize the peptic digestion products of ribonuclease, especially with respect to enzymic activity. It will be shown that the first large digestion product (obtained at pH 2.0 and 25°) is PIR. In the following paper we report the results of experiments designed to identify the abnormal tyrosyl residue of PIR.

Experimental

Materials

Ribonuclease was purchased as the five-times crystallized material from Sigma Chemical Co. (lots R42B-093 and R112B-070). The ribonuclease A fraction was prepared as described previously (Rupley and Scheraga, 1963) and was demonstrated to be chromatographically pure. Ribonucleic acid was purchased from Nutritional Biochemicals Corp. Analytical grade Bio-Rex 70 resin (200–400 mesh) from Bio-Rad Laboratories was washed twice with 6 N HCl and then washed thoroughly with water. Twice crystallized pepsin (lot 681) and carboxypeptidase A [diisopropylphosphorofluoridate (DFP) treated] were obtained from Worthing-

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[1] Abbreviation used: PIR, pepsin-inactivated ribonuclease.

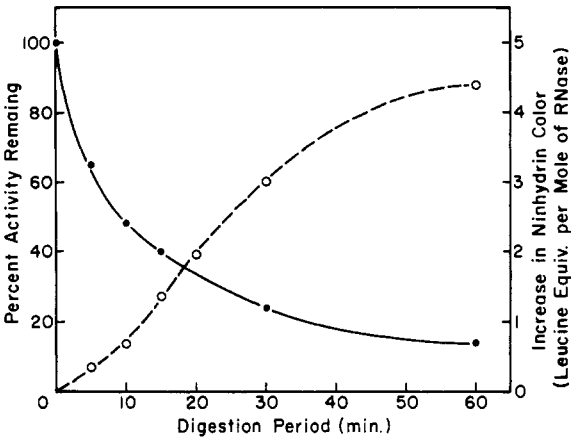

FIGURE 1: Peptic digestion of ribonuclease A at pH 2.0 (dilute HCl) and at 25°. The initial concentration of ribonuclease A was 10 mg/ml; that of pepsin was 7.4 μg/ml. The solid circles represent the enzymic activity of the digestion mixture, and the open circles the increase in ninhydrin color.

ton Biochemical Corp. 1-Fluoro-2,4-dinitrobenzene and trimethylamine were Eastman Kodak White Label products. Sephadex G-75 (medium; Pharmacia) was washed and packed into a column of 1.0 × 140 cm according to Crestfield *et al.* (1962). All other reagents were either reagent grade or the best grade available.

Methods

Digestion of ribonuclease A by pepsin was carried out at pH 2.0 (dilute HCl) and at 25°. Concentrations of ribonuclease A of 10 mg/ml and pepsin of 7 μg/ml were used routinely. No change in pH of the reaction mixture was observed for 10 minutes. However, for longer digestion times it was necessary to add HCl to maintain the pH constant. At various stages of the digestion, 0.1-ml aliquots of the solution were pipetted into test tubes containing 4 ml of 0.1 M sodium phosphate buffer, pH 7.4, to terminate the digestion, and enzymic activity and increase in ninhydrin color were measured. For ultimate use in analytical column chromatography, 1 ml of the digestion mixture was added to 0.5 ml of the pH 7.4 buffer, which had been cooled to 0°, and then quickly frozen.

Chromatographic Analyses of Digestion Mixture. Analytical chromatography was carried out at room temperature on 0.9 × 30 cm columns of Bio-Rex 70 in conjunction with a Technicon Autoanalyzer. The columns were equilibrated with 0.2 M sodium phosphate and 0.001 M sodium Versenate buffer, pH 6.4. A gradient in the eluent buffer was produced with a Technicon Autograd, in which the first three chambers contained 35 ml each of 0.2 M sodium phosphate and 0.001 M sodium Versenate buffer, pH 6.4, and the fourth chamber contained 35 ml of 0.8 M sodium phosphate and 0.001 M sodium Versenate buffer, pH 6.2. The ninhydrin color value of the effluent (flow rate, about 20 ml/hour) was obtained with the aid of a Technicon Autoanalyzer.

Preparative chromatography (100 mg to 1 g of protein) was performed on larger Bio-Rex 70 columns (1.4 × 30 to 2.9 × 50 cm) using larger volumes of the eluent buffers (80–350 ml in each Autograd chamber), with a greater flow rate of 35–70 ml/hour. The protein fractions were pooled and desalted by gel filtration on a 2.8 × 30 cm column of Sephadex G-25 with 0.05 M ammonium acetate as supporting electrolyte, followed by repeated lyophilization. In a similar fashion, the various tubes in each *peptide* fraction were pooled and lyophilized.

Ribonuclease activity was measured by the spectrophotometric method of Kunitz (1946) using ribonucleic acid as substrate (Rupley and Scheraga, 1963). *Performic acid oxidation* was carried out at −10° according to Hirs (1956). *Amino acid analyses* were performed with a Technicon amino acid analyzer. All hydrolyses were carried out in 6 N HCl in evacuated and sealed ampoules for 22 hours at 110°. The data on the proteins were obtained by assuming the theoretical numbers for the following residues in order to calculate the average number of micromoles of the protein in the hydrolysate (the average number of micromoles of the amino acid divided by the theoretical number of the respective amino acid): aspartic acid, glutamic acid, glycine, alanine, phenylalanine, and arginine. The numbers of residues per molecule were then calculated for all the amino acids using the amount of the protein thus determined. This procedure was necessary since the concentration of the sample could not be determined accurately because of the small quantities of proteins hydrolyzed. Corrections for hydrolysis losses were applied for serine (89%), threonine (95%), tyrosine (85%), and cystine (81%) (Gundlach *et al.*, 1959), and for cysteic acid (84%) (Rupley and Scheraga, 1963).

Reaction with fluorodinitrobenzene was carried out according to Hirs (1956), using trimethylamine as buffer. Dinitrophenylated samples were hydrolyzed for 16 hours at 110° in evacuated and sealed ampoules. Paper chromatography of the ether-soluble dinitrophenyl (DNP) derivatives was developed according to Levy (Fraenkel-Conrat *et al.*, 1955). The recovery of di-DNP-lysine from ribonuclease A was about 70%, and taken as a reference for corrections of N-terminal di-DNP-lysine from the other proteins. The recoveries of other DNP-amino acids from PIR-2 and PIR-3 (these designations will be described in the next section) were assumed to be the same as that of di-DNP-lysine (*i.e.*, 70%). The molecular weights of the intermediates were assumed to be the following: PIR-1, 13,300 (ribonuclease A minus the C-terminal tetrapeptide); PIR-2, 12,700 (PIR-1 minus 0.5 mole of decapeptide (46–55); the decapeptide was not quantitatively released); PIR-3, 11,100 (PIR-1 minus decapeptide (46–55) minus octapeptide (1–8)). Qualitative identification of the N-terminal residue of the tetrapeptide peak was also made using the *t*-amyl alcohol–phthalate buffer, pH 6.0, system of Blackburn and Lowther (1951).

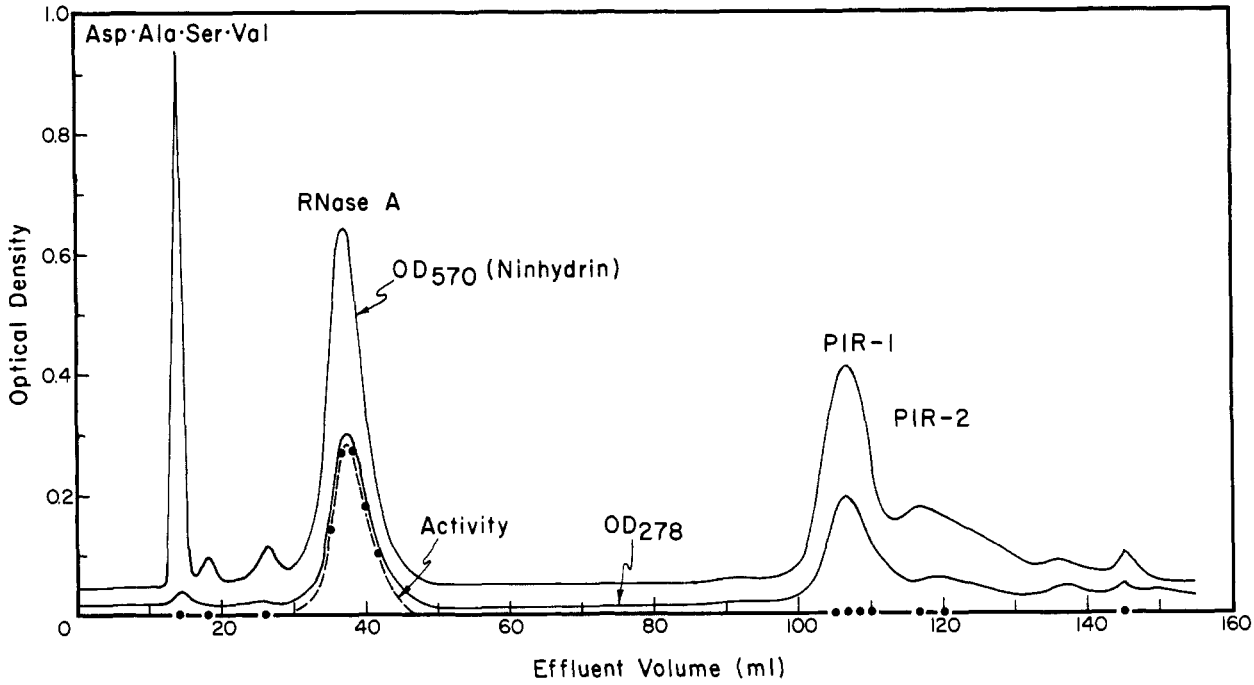

FIGURE 2: Chromatography of a peptic digest of ribonuclease A (pH 2.0, 25°, 10 minutes) on a 0.9 × 30 cm Bio-Rex 70 column. The load was 25 mg. One-third of the effluent solution was used for the development of ninhydrin color and analyzed with a Technicon Autoanalyzer, and two-thirds was collected for the measurement of optical density at 278 mμ and enzymic activity (closed circles), the latter being expressed as concentration of ribonuclease A in terms of optical density at 278 mμ.

Carboxypeptidase A Digestion of Proteins. Native or oxidized protein (3–4 mg/ml) was incubated with the enzyme (molar ratio, 1:250–400) in 0.2 M NaCl, 0.1 M Tris buffer, pH 8.0, at 40° for 3 hours. The digestion mixture was then transferred into a test tube containing 0.2 N HCl and immediately frozen. Amino acids released by carboxypeptidase A were analyzed with a Technicon amino acid analyzer.

Gel Filtration of Oxidized Proteins on a Sephadex G-75 Column. The Sephadex column of 1.0 × 140 cm was equilibrated with 0.2 M sodium phosphate buffer, pH 6.47, at room temperature. Oxidized protein (4–5 mg) was dissolved in 1 ml of the buffer and run on the column at a flow rate of about 3 ml/hour and at room temperature. Fractions (3 ml) were collected and analyzed by the ninhydrin reaction with the aid of a Technicon Autoanalyzer. The column was reused after overnight washing with the same buffer.

Spectrophotometric titration measurements were made at a wavelength of 295 mμ using a Beckman Model DU spectrophotometer. The temperature of the cell compartment was maintained at 25 or 5° by circulating water from a constant-temperature bath. pH was measured with a Beckman Model G pH meter. The protein solutions in 0.15 M KCl were prepared at a concentration of 0.33–0.65 mg/ml. The pH of the solution was changed by additions of small amounts of NaOH or HCl solution.

Results

Peptic Digestion of Ribonuclease A at 25° and at pH 2.0. The course of the digestion was measured by the increase in ninhydrin color and by the loss of enzymic activity. In Figure 1, the extent of hydrolysis was calculated from the increase in ninhydrin color of the digestion mixture (as increase in leucine equivalent per mole of ribonuclease A). The observed rapid inactivation of ribonuclease A by pepsin was in agreement with the results of Anfinsen (1956); when 0.7 mole of the peptide bond had been split, the activity of the digestion mixture decreased to 50%. The digestion mixture had almost no activity after 1 hour, when 4–5 bonds had been split; this is nearly one-half of the number of bonds of oxidized ribonuclease which may be cleaved by pepsin (Bailey *et al.*, 1956).

Chromatographic Separation of the Digestion Products. The gradient conditions used for chromatography on a Bio-Rex 70 column were chosen to produce an optimum separation of the high molecular weight digestion products. Figure 2 shows a chromatographic pattern from a 10-minute digest. In this experiment, one-third of the effluent solution was continuously developed for ninhydrin color and analyzed with a Technicon Autoanalyzer (OD_{570}), and two-thirds was collected for measurement of optical density at 278 mμ (OD_{278}) and activity toward ribonucleic acid, the latter being expressed as concentration of ribonuclease A (OD_{278}).

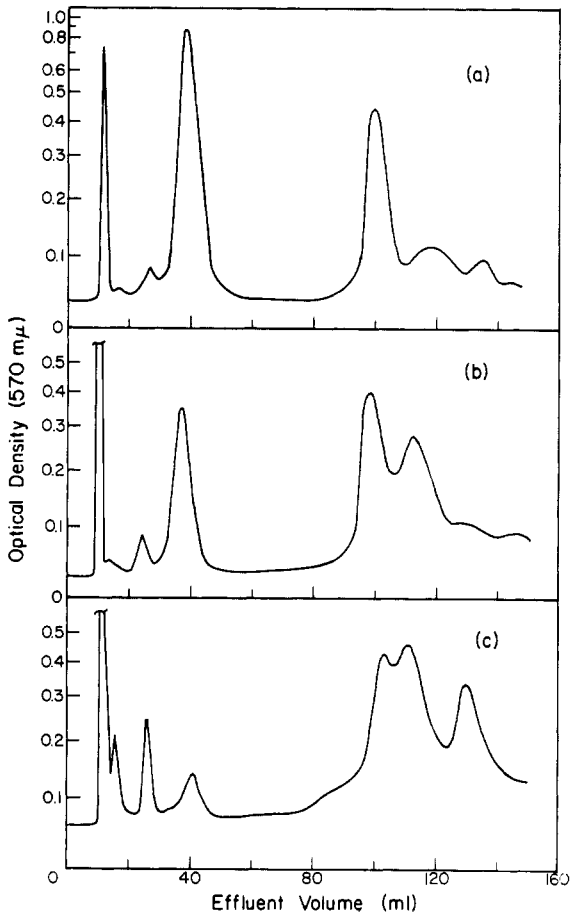

FIGURE 3: Chromatographic analyses of peptic digests of ribonuclease A (pH 2.0, 25°) on a 0.9 × 30 cm Bio-Rex 70 column. The load was 7 mg for all three runs. The ninhydrin color value of the effluent (flow rate, 20 ml/hour) was obtained with the aid of a Technicon Autoanalyzer: (a) 5.5 minutes (about 0.4 bond split), (b) 20.5 minutes (about 1.3 bonds split), and (c) 60 minutes (about 4.4 bonds split).

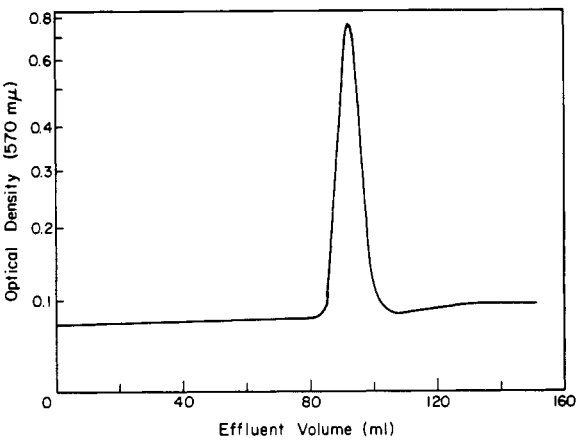

FIGURE 4: Analytical chromatography of the PIR-1 peak of Figure 2 on a 0.9 × 30 cm Bio-Rex 70 column. The ninhydrin color value of the effluent (flow rate, 20 ml/hour) was obtained with the aid of a Technicon Autoanalyzer.

The fast-moving peak was assumed to be a low molecular weight peptide peak. The second large peak in the chromatogram seems to be unreacted ribonuclease A from the position on the chromatogram and from its full activity. This component will be designated as peak "A" in the following sections. Among the other inactive peaks which were eluted in the later stages of chromatography, the larger two peaks will be designated as PIR-1 and PIR-2, respectively, in order to differentiate them for the moment from Anfinsen's PIR (1956).

Chromatographic analyses of the digestion products at various digestion periods are shown in Figure 3. As the digestion proceeded, the heights of the fast-moving peaks and the slow-moving peaks (including PIR-1 and PIR-2) increased at the expense of peak A. After 60 minutes (Figure 3c), almost all of the ribonuclease A was digested to inactive components. After exhaustive digestion (17 hours), three large fast-moving peaks were obtained, followed by broad peaks which were eluted in the later stages of chromatography and not separated with the gradient conditions used here. No attempts were made to analyze these slow-moving peaks.

Digestion at Various Temperatures. Because Anfinsen (1956) isolated PIR by digestion of ribonuclease at 37°, it was of interest to compare the course of the digestion at different temperatures. Digestion was carried out for 10 minutes at pH 2.0 and at 0, 10, 15, 20, 25, 30, 35, and 40°. Chromatographic analyses of the digestion products showed that, as the temperature increased, the quantities of the fast-moving peptide peaks and slow-moving inactive components increased at the expense of peak A. Above 25° the height of the PIR-2 peak increased as the temperature increased, but that of the PIR-1 peak did not change much. No new peaks, other than those found in the digest at 25°, were observed in any of the digests at other temperatures. Therefore, it was concluded that the course of the early stages of peptic digestion is probably independent of temperature up to 40°, even though the ribonuclease A molecule is unfolded to a larger degree and the rate of hydrolysis is greater at higher temperatures. Thus digestion at 25° for a rather short period (10 minutes) was considered preferable in order to prepare PIR in a good yield, since the PIR-1 peak was demonstrated (see below) to be identical with Anfinsen's PIR.

Fractionation of Digestion Products. The components of preparative-scale digestion mixtures (25°, 10 minutes for preparation of PIR-1 and PIR-2) were separated on Bio-Rex 70, and the protein fractions were combined and desalted on Sephadex G-25 followed by lyophilization. On analytical chromatography, peak A

TABLE I: Amino Acid Analyses of Components of Peptic Digest of Ribonuclease A.[a]

Amino Acid	Ribonuclease A		Peak	PIR-1			PIR-2[c]	PIR-3[c]	Tetra-peptide Peak (Molar Ratio)	Octa-peptide Peak (Molar Ratio)
	Theory	Exptl[b]	A[b]	Theory	Exptl[b]	Oxidized[b]				
Cysteic acid				(8)		7.5				
Methionine sulfone				(4)		4.0				
Aspartic acid	15	15.1	15.0	14	14.1	13.8	13.4	13.5	1.06	0.36
Threonine	10	10.1	10.3	10	10.3	10.3	10.3	8.7		1.1
Serine	15	14.8	14.8	14	14.2	13.9	13.5	13.0	1.0	0.36
Glutamic acid	12	12.3	12.3	12	12.5	12.1	11.2	10.5	0.14	1.1
Proline	4	4.48	4.46	4	4.45	4.48	4.6	4.5		
Glycine	3	3.04	3.02	3	3.05	3.08	3.03	2.92		
Alanine	12	12.0	12.1	11	11.0	10.9	10.4	8.18	1.0[d]	2.6
Half-cystine	8	8.4	7.6	8	8.20		7.5	7.9		
Valine	9	8.98	8.83	8	7.80	7.89	6.66	6.83	1.03	0.31
Methionine	4	4.0	3.54	4	3.64		3.55	3.38		
Isoleucine	3	2.11	2.11	3	2.06	2.15	1.98	1.93		
Leucine	2	1.99	2.02	2	1.99	2.06	1.18	1.24	0.07	
Tyrosine	6	6.01	6.16	6	6.31	6.22	5.88	6.30		
Phenylalanine	3	3.00	3.03	3	3.01	3.00	2.19	1.36	0.06	0.96
Lysine	10	10.2	10.4	10	9.91	10.0	10.1	7.95		2.0[d]
Histidine	4	3.92	3.95	4	3.80	3.82	3.06	2.97	0.07	
Arginine	4	4.01	3.82	4	3.94	3.97	3.96	4.08		

[a] Moles of amino acid per mole of protein. [b] For ribonuclease A, peak A, and PIR-1, the average of the theoretical numbers of the following amino acids were used for calculation: aspartic acid, glutamic acid, glycine, alanine, phenylalanine, and arginine. [c] For PIR-2 and PIR-3, the average of the theoretical numbers of glycine and arginine were used for calculation. [d] Assumed as reference.

was superimposable with native ribonuclease A, and had full activity toward ribonucleic acid. PIR-1 (Figure 4) was chromatographically pure, while the PIR-2 peak (Figure 5a) was asymmetrical. These two derivatives had no activity toward ribonucleic acid.

In an experiment on a 60-minute digest, the high molecular weight inactive component, which was eluted before the PIR-1 peak (see Figure 3c) and had no activity, was also recovered for chemical analyses. This component was designated as PIR-3 and its chromatographic pattern (Figure 5b) showed its inhomogeneity.

The peptide fractions, the tetrapeptide fraction shown in Figure 2, and the component which was eluted before peak A in Figure 3c were lyophilized without desalting.

Amino Acid Analyses of Digestion Products. The amino acid compositions of the digestion products as well as of ribonuclease A are listed in Table I. The analyses for ribonuclease A and peak A agreed with theory, supporting the conclusion based on the chromatographical identity between these two species. PIR-1 and performic acid oxidized PIR-1 had the composition for the species obtained by removal of the C-terminal tetrapeptide (residues 121–124) of ribonuclease A. Several preparations of PIR-1 showed the same analytical results within the experimental error (±4%). In addition, the main component of the fast-moving peptide peak (the tetrapeptide peak in Table I) was found to have the composition of the tetrapeptide, *i.e.*, approximately equimolar quantities of aspartic acid, serine, alanine, and valine. PIR-2 had a similar composition to that of PIR-1 except for lower values for several amino acids (although less than one residue each), which suggested that this component had lost a portion of an internal peptide.

The component before the PIR-1 peak, recovered from a 60-minute hydrolysate (PIR-3 in Table I), had fewer amino acid residues than PIR-2, especially alanine and lysine. On the other hand, analysis of the peptide peak before peak A (the octapeptide peak in Table I) indicated that the main component of this peak had the composition of the N-terminal octapeptide.

N- and C-Terminal Amino Acid Analyses. Table II summarizes the results of N-terminal amino acid analyses by the DNP method. Peak A and PIR-1 each had 1 mole of lysine as N terminus, indicating that these components did not have any inner peptide bond cleavage. In contrast, PIR-2 was found to have N-terminal serine and alanine as well as lysine, which might possibly be accounted for by the presence of

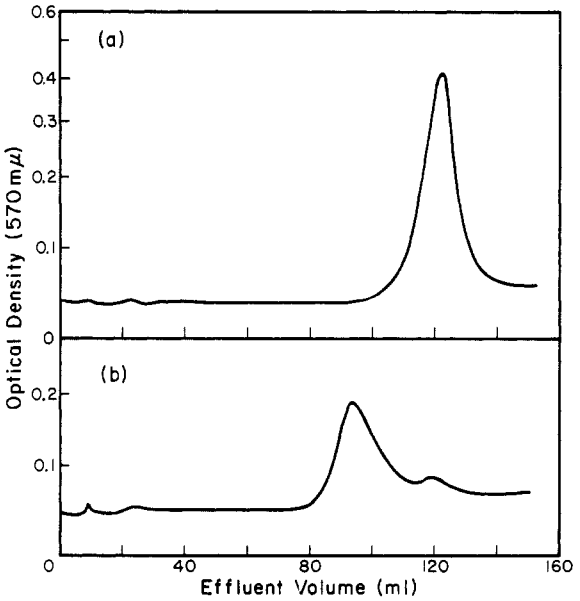

FIGURE 5: Analytical chromatography on a 0.9 × 30 cm Bio-Rex 70 column of (a) the PIR-2 peak of Figure 2 and (b) the PIR-3 peak (the component before PIR-1 in Figure 3c). The ninhydrin color value of the effluent (flow rate, 20 ml/hour) was obtained with the aid of a Technicon Autoanalyzer.

TABLE II: N-Terminal Amino Acid Analyses.[a]

DNP-Amino Acid	RNAse A	Peak A	PIR-1	PIR-2	PIR-3
Di-DNP-lysine	1.0	1.0	1.0	0.93	0.12
DNP-serine				0.63	0.43
DNP-alanine				0.33	0.7
DNP-valine				0.15	0.27
DNP-glutamic acid					0.85[b]

[a] Moles of DNP-amino acid per mole of protein; the data are expressed as molar ratios of DNP-amino acids to di-DNP-lysine recovered from ribonuclease A. The latter was assumed as reference. [b] Identified as DNP-glutamic acid by rechromatography using *t*-amyl alcohol–phthalate (Blackburn and Lowther, 1951).

inner peptide bond cleavages. The asymmetrical chromatographic pattern (Figure 5a) and N-terminal analysis data suggested that PIR-2 was heterogeneous.

In PIR-3 a new N-terminal residue, glutamic acid, was found at the expense of lysine; additional N-terminal alanine, serine, and a smaller amount of valine indicated heterogeneity of this material. The main component was assumed to have N-terminal glutamic acid and inner splits, by analogy with PIR-2.

The N-terminal residue of the main component of the tetrapeptide peak was qualitatively identified as aspartic acid. This analysis, together with the amino acid composition data, indicated that the main component was the C-terminal tetrapeptide, Asp-Ala-Ser-Val.

Analyses of amino acids released by DFP-treated carboxypeptidase A were carried out on performic acid oxidized or on native proteins. It can be seen from Table III that carboxypeptidase A released the C-terminal amino acid residues up to histidine 119 from both oxidized ribonuclease A and oxidized peak A. This confirmed the conclusion deduced above that peak A was unreacted ribonuclease A. Native or oxidized PIR-1 yielded C-terminal phenylalanine (120) and histidine (119) in stoichiometric quantities. PIR-2 showed C-terminal phenylalanine followed by histidine, as well as other amino acids, as expected from the suggested presence of inner splits. Identification of the C-terminal residues of PIR-2 will be discussed later. Because of the limited amount of PIR-3, carboxypeptidase digestion could not be carried out with this component.

TABLE III: Amino Acids Released by Carboxypeptidase A.[a]

Amino Acid	Oxidized RNAase A	Oxidized Peak A	Oxidized PIR-1	Oxidized PIR-1	Oxidized PIR-2
Aspartic acid	0.78	0.65			
Threonine					0.80
Serine	0.86	0.86			0.51
Alanine	0.79	0.68			
Valine	1.0	1.0			
Methionine					0.72
Phenylalanine	0.79	0.70	0.90	0.96	0.96
Tyrosine					0.55
Histidine	0.64	0.56	0.84	0.89	0.89

[a] Moles of amino acid per mole of protein.

Gel Filtration of Oxidized Proteins on a Sephadex G-75 Column. In order to demonstrate that the PIR-1 molecule has no inner split, gel filtration of oxidized PIR-1 on Sephadex G-75 was carried out; similar experiments were performed with oxidized ribonuclease A and oxidized PIR-2. If PIR-1 has no inner split, the oxidized molecule should behave on the column in the same manner as oxidized ribonuclease A, since the two molecules differ only in the C-terminal tetrapeptide. From Figure 6a and 6b, it is clear that the two elution patterns were the same except that oxidized PIR-1 showed a negligible amount of a component of smaller size. On the other hand, the behavior of oxidized PIR-2 (Figure 6c) was quite different from the others, showing two major peaks of smaller size than that obtained for PIR-1 and ribonuclease A. These experi-

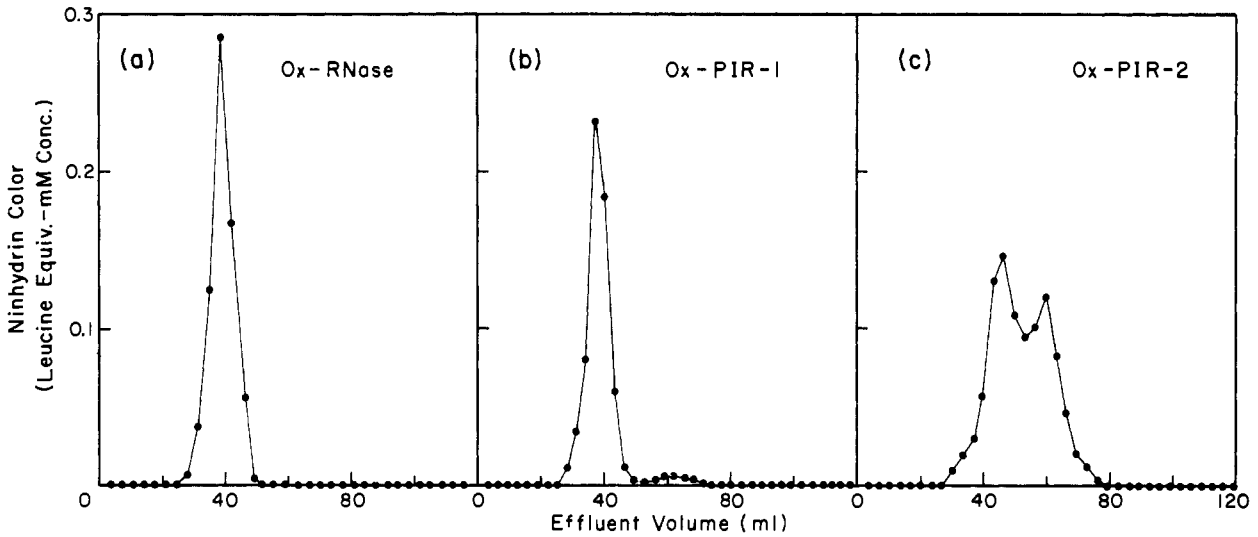

FIGURE 6: Behavior of oxidized proteins on a 1.0 × 140 cm Sephadex G-75 column. The buffer was 0.2 M sodium phosphate, pH 6.47, at room temperature, at a flow rate of 3 ml/hour. Fractions (3 ml) were collected and analyzed by the ninhydrin reaction with the aid of a Technicon Autoanalyzer: (a) 5 mg of oxidized ribonuclease A, (b) 4 mg of oxidized PIR-1, and (c) 5 mg of oxidized PIR-2.

ments supported the conclusion that PIR-2 has inner splits, whereas PIR-1 does not.

Oxidized peak A was also subjected to gel filtration; the elution pattern was the same as that obtained for oxidized ribonuclease A (Figure 6a). This confirmed the conclusion that peak A is unreacted ribonuclease A.

Spectrophotmetric Titration of PIR-1. The abnormal tyrosine in PIR-1 (Bigelow and Ottesen, 1957) was studied by spectrophotometric titrations at 295 mμ. The titration curves of native and oxidized PIR-1 are presented in Figure 7. Oxidized PIR-1 had six normal tyrosyl groups as expected from its disordered conformation in aqueous solution. The total increase in optical density at 295 mμ corresponded to an increase in molar extinction coefficient ($\Delta\epsilon$) of 15,600, thus giving $\Delta\epsilon$ 2600 for each tyrosyl residue. Native PIR-1 was found to contain one abnormal tyrosine which ionizes irreversibly above pH 12.5. This observation is consistent with the normalization of two of the three abnormal tyrosines present in ribonuclease (Shugar, 1952; Tanford *et al.*, 1955) reported previously by Bigelow and Ottesen (1959) for Anfinsen's PIR.

From the apparent dissociation constants (pK) of the normal tyrosyl groups of PIR-1 at 25 and 5°, the apparent heat of ionization, ΔH, was calculated with the aid of the equation

$$\Delta H = 2.303 \text{ d p}K/\text{d } (1/T)$$

A value of about 5.7 kcal/mole was obtained; this agrees with the value of 5.5 kcal/mole for ΔH of the normal tyrosine ionization of ribonuclease at ionic strength of 0.15 (Tanford *et al.*, 1955).

PIR-2 was also shown to have nearly one abnormal tyrosine by spectrophotometric titration at 25°. Because PIR-3 aggregated at high pH, successful titration experiments could not be carried out with this component.

Discussion

On the basis of the data of Tables I, II, and III, it is possible to identify the chemical structures of the various PIR derivatives. PIR-1 is a molecule in which the C-terminal tetrapeptide (residues 121–124) is missing, since its amino acid composition showed that it contained 1 mole each of aspartic acid, serine, alanine, and valine less than does ribonuclease A. In support of this result, the N-terminal residue was lysine (the N terminus of ribonuclease A) and the C-terminal residue was phenylalanine (120) followed by histidine (119). This component is, therefore, believed to be the same derivative as that which Anfinsen (1956) designated as PIR. PIR-2 is a mixture of more than one (possibly two) molecular species, both of which are missing the C-terminal tetrapeptide, one with the Met-Ser (79-80) bond split and the other with the decapeptide (residues 46–55) missing. This identification is based on (1) the comparable quantities of N-terminal serine (80) (about 0.6 mole, Table II) and C-terminal methionine (79) which was presumably followed by threonine, serine, and tyrosine (about 0.7 mole, Table III); (2) comparison in Table I of amino acid analysis data of PIR-1 and PIR-2 (the latter had less leucine, phenylalanine, and histidine than did the former, and these amino acid residues are located in positions 46, 48, and 51 of the amino acid sequence (Hirs *et al.*, 1960); (3) a rather large amount of threonine in the carboxypeptidase A digest of PIR-2 (0.8 mole, Table III) suggests an additional C-terminal

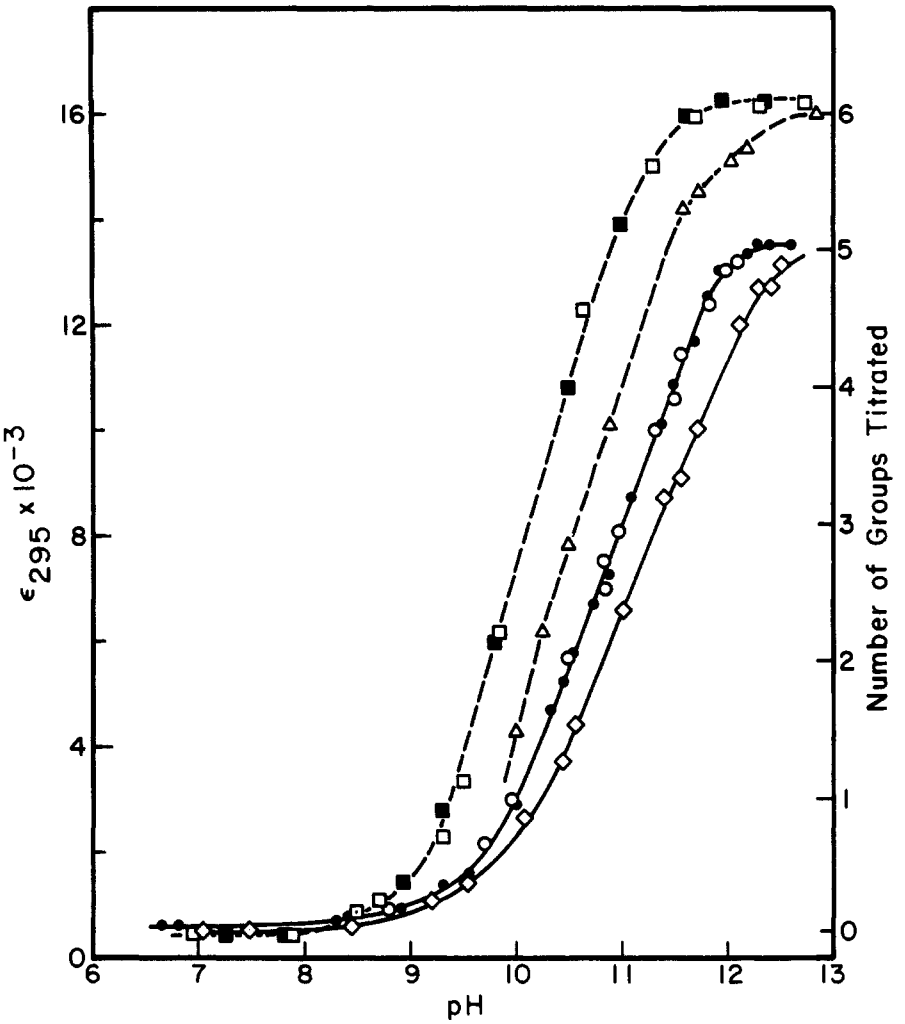

FIGURE 7: Spectrophotometric titration curves of PIR-1 and oxidized PIR-1 at 295 mμ in 0.15 M KCl: ■, forward titration of oxidized PIR-1 at 25°; □, back titration from pH 12.75; ●, forward titration of PIR-1 at 25°; ○, back titration from pH 12.5; △, back titration of PIR-1 after exposure to pH 12.8 for 1 hour at 25°; ◇, forward titration of PIR-1 at 5°.

threonine (45) in PIR-2; and (4) amino acid analysis of the tetrapeptide peak showed a minor amount of the amino acids corresponding to the sequence which includes residues 46–55. As the digestion proceeded (for example, 60 minutes), these amino acids were found in greater quantities in the tetrapeptide peak.

Another cleavage by pepsin is suggested at the Phe-Glu (8-9) bond from analytical data on the peptide peak which was eluted before peak A and on component PIR-3 which was eluted before the PIR-1 peak. This peptide peak was found to consist of the amino acids of the N-terminal octapeptide (residues 1–8, Table I); also, PIR-3 consisted of a mixture of high molecular weight intermediates, a major component of which could be accounted for by assuming that it had lost the N terminus (residues 1–8) together with the C terminus (residues 121–124) and the decapeptide (residues 46–55) (Table I). N-Terminal analysis of this component (Table II) supported this assignment, since it yielded N-terminal glutamic acid in a significantly large quantity (0.85 mole) at the expense of lysine (0.1 mole), as well as alanine (0.7 mole). The cleavage at Met-Ser (79-80) was found to a lesser degree in this component (N-terminal serine, 0.43 mole).

Since no other positions are consistent with these analytical data, we conclude that the bonds split by pepsin are Phe-Asp (120-121), Met-Ser (79-80), Thr-Phe (45-46), Glu-Ala (55-56), and Phe-Glu (8-9). The first of these bonds is most easily cleaved by pepsin, and this modification causes complete inactivation of ribonuclease A.

In acid solution, the first step of the unfolding of ribonuclease A seems to involve a neutralization of one or more carboxyl groups interacting with abnormal tyrosyl groups, giving rise to an ultraviolet difference spectrum (Scott and Scheraga, 1963). This unfolding probably renders some of the peptide bonds accessible

to peptic cleavage. Since the bond Phe-Asp (120-121) is quantitatively split in all intermediates isolated, it can be concluded that the C-terminal region of the ribonuclease A molecule is unfolded at low pH; this behavior is in contrast to the thermal denaturation of ribonuclease A at neutral pH where chymotrypsin failed to release the C-terminal tetrapeptide (Rupley and Scheraga, 1963). It was not possible to demonstrate how the subsequent cleavages depend on the degree of unfolding. Therefore, the possibility exists that the unfolding of the C terminus and the removal of the tetrapeptide lead to unfolding of other parts of the molecule.

Some interrelationship between the N- and C-terminal regions of ribonuclease has been postulated (Ottesen and Stracher, 1960). The fact that the C-terminal valine is extremely resistant to carboxypeptidase A action (Sela *et al.*, 1957) strongly suggests that the two ends of the molecule interact with each other. It may be inferred that a loosened conformation of the N-terminal region, upon removal of the C terminus, can result in an unfolding of the native structure around histidine residues 119 and 12, which are believed to lie at the active center of the enzyme (Gundlach *et al.*, 1959; Barnard and Stein, 1959; Crestfield *et al.*, 1963a,b), together with lysine 41 (Hirs, 1962; Cooke *et al.*, 1963). Changes in the conformation of ribonuclease A (in the production of PIR) are not limited to the C-terminal region of the molecule, as indicated by the normalization of two abnormal tyrosines.

Finally, Ginsburg and Schachman (1960) have studied the peptic hydrolysis of ribonuclease under similar conditions (pH 2.1–2.2, 22–26°) as those used in the present work (pH 2.0, 25°). They reported the presence of an active intermediate in the peptic digest, but they did not isolate any active component. It may be supposed that contamination by unreacted ribonuclease occurred in their experiments. In our experiments, no active intermediate with inner peptide cleavages could be identified on the chromatogram; the only active component was identified as unreacted ribonuclease A. There may be, however, some effects of ions on the course of hydrolysis, as indicated by specific anion effects on the conformation and thermal stability of ribonuclease (Crestfield and Allen, 1954; Sela *et al.*, 1957; Neumann *et al.*, 1962; Ginsburg and Carroll, 1965).

Since the abnormal tyrosine in PIR-1 may be interacting with the same carboxyl group as in native ribonuclease A, PIR-1 may be regarded as a suitable derivative to provide further information on the specific interaction between the tyrosyl and carboxyl groups in the ribonuclease A molecule (Hermans and Scheraga, 1961a,b; Scott and Scheraga, 1963). PIR-1 prepared here has been used in iodination experiments to identify the position of the abnormal tyrosine in the amino acid sequence; these experiments are reported in the following paper.

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