Structural Studies of Ribonuclease. XIX. Location of the Buried Tyrosyl Residue in Pepsin-Inactivated Ribonuclease*

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ABSTRACT: Pepsin-inactivated ribonuclease (PIR) was subjected to iodination with 12 moles of I_2/mole of PIR at pH 9.5 and at 0°. A major component of the iodination product was fractionated by chromatography on Bio-Rex 70. Amino acid analyses and spectrophotometric titration of the iodinated material showed the presence of about 1 mole of uniodinated and still abnormal tyrosine. Its ultraviolet spectrum suggested the modification of tyrosyl groups either to monoiodotyrosyl or to 3,5-diiodotyrosyl groups. The simultaneous modification of about 1 mole of histidine was shown by the amino acid analysis data. However, the iodination product of histidine was not identified. Oxidized iodinated PIR was then digested with the proteolytic enzymes trypsin, pepsin, and chymotrypsin, and the resulting peptides were chromatographed on Dowex 50-X2.

Although there are difficulties about the analyses of the peptides containing tyrosine 115 due to the simultaneous modification of histidine 105 and/or 119 and of the peptides containing tyrosine 92, comparison of the elution patterns and amino acid compositions of the peptides indicated that the *single* uniodinated tyrosyl group of PIR is residue 25.

It may be possible to determine the conformation of protein molecules in aqueous solution by regarding the molecule as a combination of cyclic structures, and computing the conformation of the cyclic portions (Némethy and Scheraga, 1965). For this purpose disulfide bridges, as well as noncovalent interactions, serve to define the cyclic structures within the molecule. In the case of ribonuclease, the positions of the four disulfide bridges are known (Spackman *et al.*, 1960), and several tyrosyl–carboxyl interactions have been proposed (Hermans and Scheraga, 1961a,b; Scott and Scheraga, 1963). Two of the three "buried" tyrosyl (Cha and Scheraga, 1963a,b; Donovan, 1963) and the three "buried" carboxyl groups (Broomfield *et al.*, 1965; Riehm *et al.*, 1965) have been identified; experiments to locate the third "buried" tyrosyl group are in progress in our laboratory.

The important problem of determining which tyrosyl groups are paired with which carboxyl groups remains. For this purpose, pepsin-inactivated ribonuclease (PIR)[1] is a suitable derivative for investigation, since it contains only one, instead of three, abnormal tyrosyl groups (Bigelow and Ottesen, 1959), and it is assumed that this single tyrosyl residue is one of the abnormal groups of native ribonuclease (Bigelow, 1961).

In the previous paper (Fujioka and Scheraga, 1965) we described the preparation and characterization of several peptic digestion products from ribonuclease. One of these, PIR-1, is identical with Anfinsen's (1956) PIR, and we shall use his designation in this paper. Our purpose here is to identify the single abnormal tyrosyl residue of PIR. This was accomplished by iodinating the five "exposed" tyrosyl groups under conditions where the "buried" tyrosyl group does not react. Amino acid analyses and spectral studies were used to determine the number of uniodinated tyrosyl groups in iodinated PIR. Peptide analyses of the proteolytic hydrolysates of oxidized iodinated PIR served to determine the position of the uniodinated tyrosyl group in the amino acid sequence. In chromatographic analyses of these peptides, those which contained the uniodinated tyrosyl group appeared in the same positions in the chromatogram as those peptides from oxidized uniodinated PIR. The peptides which contained modified tyrosyl groups disappeared from the positions in the chromatogram where the corresponding peptides from oxidized PIR appeared; one of the modified peptides in the tryptic digest could be identified in a different position in the chromatogram.

Experimental

Materials

The preparation of PIR was described in the preceding paper. It was shown to be chromatographically homogeneous and to have only one N-terminal residue (lysine).

The iodination reagent was 0.1 N in I_2 and 0.2 M in

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[1] Abbreviations used: PIR, pepsin-inactivated ribonuclease; Ox-PIR, oxidized PIR.

KI. The iodine concentration was determined by titration against standard As_2O_3 (Mallinckrodt Chemical Works).

Crystalline salt-free trypsin, crystalline salt-free chymotrypsin (lot CDI 678–84B), and twice-crystallized pepsin (lot 681) were purchased from Worthington Biochemical Corp. Monoiodotyrosine was obtained from Nutritional Biochemicals Corp., 3,5-diiodo-L-tyrosine dihydrate from Mann Research Laboratories, Inc. (Mann Assayed), and glycine (ammonia free) from Eastman Kodak Co. Analytical grade Dowex 50-X2 resin from Bio-Rad Laboratories was prepared according to the procedure of Hirs *et al.* (1956a). All other chemicals were either reagent grade or the best grade available.

Methods

Iodination of PIR was carried out according to the procedure of Hughes and Straessle (1950) and Cha and Scheraga (1963a). The conditions are reported to be specific for iodination of tyrosyl residues in proteins. PIR (200 mg) was dissolved in 10 ml of 0.5 M glycine buffer, pH 9.5. The solution was then cooled to 0° and stirred magnetically. Approximately 12 moles of I_2/mole of PIR was added dropwise to the protein solution over a period of 1 hour, using a micropipet. The reaction was allowed to proceed for 4 hours at 0°. The yellow color due to the presence of excess iodine persisted. Removal of glycine, KI, and I_2 was then accomplished by gel filtration on a 2.8 × 30 cm column of Sephadex G-25 in the cold room (4°). Protein fractions from several tubes were combined and repeatedly lyophilized. The yield was about 150 mg.

Oxidized PIR (Ox-PIR) was iodinated under the same conditions as described above. The iodinated material was subjected to gel filtration, then lyophilized and used in some of the experiments described later to see the differences between the iodinated tyrosyl groups in PIR and Ox-PIR.

Fractionation of Iodinated Product. The iodinated material was purified by chromatography on Bio-Rex 70. The procedure was the same as that for the preparation of PIR described in the preceding paper. A column of 2.5 × 40 cm and a total volume of eluent buffer of 1 liter were routinely used with a flow rate of about 100 ml/hour for chromatography of 150 mg of iodinated material. The iodinated protein did not dissolve easily in water, and it took a long time to make a viscous emulsion which was then put on the column. Fractions (10 ml) were collected and the optical density at 278 mμ was measured. The protein fractions (from several tubes) were pooled, desalted on Sephadex G-25, and lyophilized. The yield was about 50 mg.

Analytical chromatography on Bio-Rex 70 was performed on 0.9 × 30 cm columns of Bio-Rex 70 in conjunction with a Technicon Autoanalyzer as described in the preceding paper.

Performic acid oxidation was carried out at −10° according to the procedure described by Hirs (1956).

Amino acid analyses of iodinated proteins were carried out on both unoxidized and oxidized preparations. The correction factors for hydrolysis losses, determined by Gundlach *et al.* (1959) and Rupley and Scheraga (1963), were used for threonine, serine, cystine, tyrosine, and cysteic acid. The procedure and calculation of the compositions were the same as described in the preceding paper.

Spectral studies were performed with a Beckman Model DU spectrophotometer. Ultraviolet spectra of iodinated PIR and iodinated Ox-PIR were measured in 0.01 M borate buffer, pH 10.5. Spectrophotometric titration was carried out with both proteins as well as with mono-and 3,5-diiodotyrosine in 0.15 M KCl at 25°. Protein concentrations were 0.47–0.6 mg/ml in the titration experiments.

Proteolytic digestion of oxidized proteins was performed at room temperature for 22 hours. Ox-PIR or oxidized iodinated PIR (15–20 mg) was digested with trypsin or chymotrypsin in 0.2 M sodium phosphate buffer, pH 7.0, or with pepsin in 0.2 M sodium citrate buffer, pH 1.9, as described by Bailey *et al.* (1956), Hirs *et al.* (1956a,b), and Cha and Scheraga (1963b). The reaction was terminated by adjusting the pH of the solution, and the digestion mixture was immediately subjected to column chromatography for peptide analyses.

Dowex 50-X2 Column Chromatography. The procedures for chromatography of the proteolytic digests of the oxidized proteins were almost identical with those described by Cha and Scheraga (1963b), except that the gradient for the tryptic digests was begun after 300 ml of the starting buffer (0.2 N citrate, buffer, pH 3.1) had been eluted instead of 200 ml, in order to obtain a better separation between O-T-2[2] and O-T-4 in the digest, as noted by Riehm *et al.* (1965). Fractions (2 ml) of the effluent were collected and analyzed for ninhydrin color to detect peptides with the aid of a Technicon Autoanalyzer. The alkaline hydrolysis of tubes 50–110 was carried out to detect the ninhydrin-negative peptide in the chymotryptic hydrolysate (O-C-2).

Amino Acid Analyses of Peptides. The appropriate peptide fractions were combined and evaporated. The residue was dissolved in water, made up to 6 N in HCl, and hydrolyzed at 110° for 22 hours in an evacuated and sealed ampoule. All hydrolyses were carried out without desalting. To correct for hydrolysis losses of amino acids in the presence of excess buffer salt, the standard amino acid solution (California Corp. for Biochemical Research) contaning 0.5 μmole each of the amino acids was added to 10 ml of 0.2 N citrate buffer, pH 3.1, or 2 N citrate–acetate buffer, pH 5.1, and subjected to hydrolysis in the same manner as described above for the peptide fractions. The following recoveries of amino acids were obtained for the two buffers: threonine, 93 and 89%; serine, 88 and 73%; and tyrosine, 60 and 60%. The amino acids other than these three were found to be stable during hydrolysis. The corrections for losses

[2] The terminology used for the peptides in the proteolytic digests in this paper is that of Hirs *et al.* (1956a) for the tryptic peptides, of Bailey *et al.* (1956) for the peptic peptides, and of Hirs *et al.* (1956b) for the chymotryptic peptides.

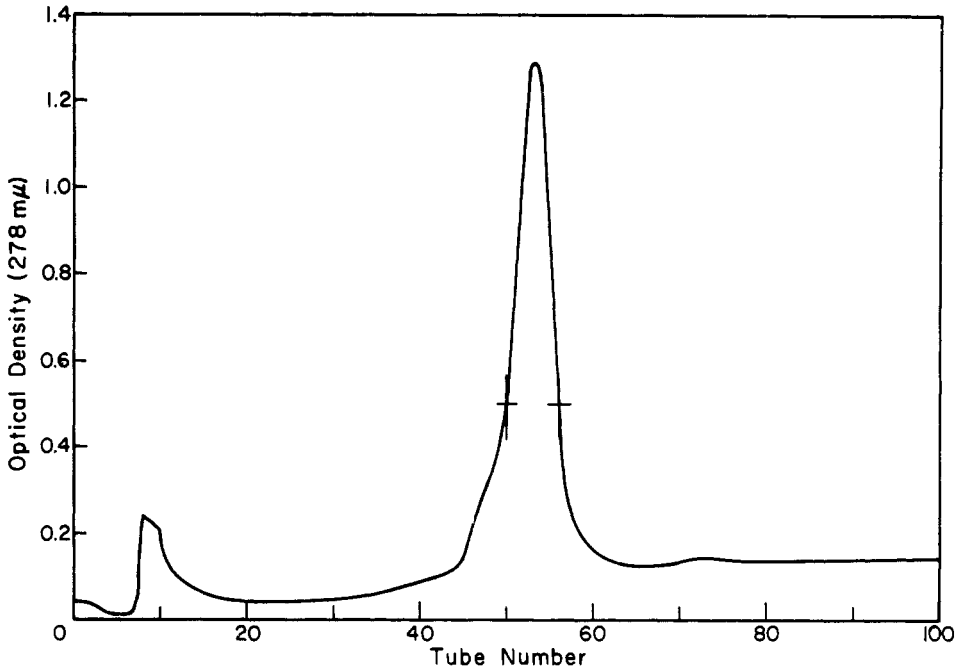

FIGURE 1: Chromatographic separation of iodinated PIR (150 mg) on a 2.5 × 40 cm Bio-Rex 70 column. The effluent (flow rate of about 100 ml/hour) was collected in 10-ml fractions. The protein fractions (tube numbers 50–56) were combined and freed of buffer salts by gel filtration on Sephadex G-25. This preparation is sample A.

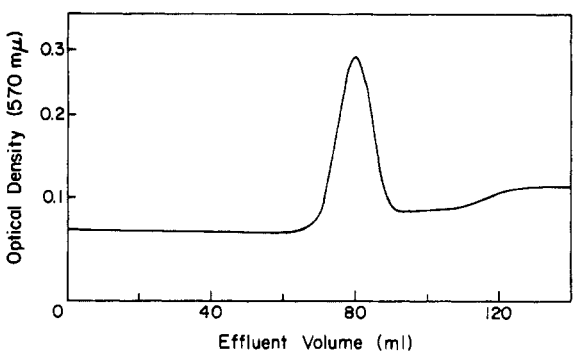

FIGURE 2: Analytical chromatography of iodinated and fractionated PIR (sample A of Figure 1) on a 0.9 × 30 cm Bio-Rex 70 column. The load was 2 mg. The ninhydrin color value of the effluent (flow rate, 20 ml/hour) was obtained with the aid of a Technicon Auto-analyzer (occasionally a ninhydrin-positive impurity in the phosphate buffer contributed to the base line).

of these three amino acids were applied to the analytical data for the peptides eluted on the first half of the chromatograms (O-T-2, O-T-4, O-P-5, O-C-2, O-C-3, O-C-11, O-C-12, and O-C-14), using correction factors based on the presence of 0.2 N citrate buffer. For the second half of the chromatograms (O-T-14, O-C-22, O-C-29, and O-C-30), the correction factors were based on the presence of 2 N citrate–acetate buffer. The amino acid composition of each peptide was calculated by assuming the theoretical value for the number of an appropriate amino acid residue and using this value to determine the number of residues for the other amino acids.

The yield of peptides was calculated on the basis of a molecular weight of 13,800 for the oxidized protein (13,300 + O_{32} + H_8) and an assumed moisture content of 10% for the lyophilized samples.

Results

Chromatographic Separation of Iodinated PIR. A chromatographic pattern for a preparative-scale fractionation of the iodinated material is shown in Figure 1. In contrast to the iodination of ribonuclease A (Cha and Scheraga, 1963a), the iodination product (12 moles of I_2/mole of PIR) did not separate into many fractions on Bio-Rex 70 at pH 6.4. Several gradient conditions were used, but no better resolution of the product could be obtained. Ideally, chromatography of the iodinated product should be carried out at a pH value between the pK values of mono- and 3,5-diiodotyrosine (see Table II), since such a condition would result in resolution between the derivatives which differ only slightly in the numbers of iodotyrosines. However, chromatographic separation on CM-cellulose at pH 8.5, which has been shown to be useful for the separation of the iodination product of ribonuclease A,[3] was not successful.

Analytical chromatography of the iodinated and

[3] Woody, R. R., and Scheraga, H. A., unpublished data.

TABLE I: Amino Acid Analyses of Iodinated PIR.[a]

Amino Acid	Ox-PIR Theory	Ox-PIR Experiment	Iodinated PIR Sample A (before oxidation)	Iodinated PIR Sample A (oxidized)	Iodinated PIR Sample B (oxidized)	Iodinated Ox-PIR (before oxidation[b])	Iodinated Ox-PIR (oxidized[b])
Cysteic acid	8	7.5		7.55	8.00	8.26	8.22
Methionine sulfone	4	4.0		3.63	3.98	4.00	4.06
Aspartic acid	14	13.8	13.8	14.2	14.3	13.9	13.9
Threonine	10	10.3	10.5	9.5	10.1	10.1	10.1
Serine	14	13.9	13.7	13.8	13.6	14.3	13.8
Glutamic acid	12	12.1	11.9	12.0	12.4	12.0	12.1
Proline	4	4.48	3.82	3.97	4.66	3.93	3.6
Glycine	3	3.08	3.22	3.02	3.25	3.9[c]	3.7[c]
Alanine	11	10.9	11.1	11.1	11.2	11.2	10.8
Half-cystine			8.34				
Valine	8	7.89	7.81	7.73	8.08	7.75	7.65
Methionine			3.93				
Isoleucine	3	2.15	1.97	2.23	2.16	2.07	2.03
Leucine	2	2.06	2.01	2.14	2.00	2.06	2.13
Tyrosine	6	6.22	5.71[d]	1.26	1.40	4.23[d]	0.2
Phenylalanine	3	3.00	3.00	3.03	2.94	2.92	2.89
Lysine	10	10.0	10.1	9.90	9.44	10.3	10.3
Histidine	4	3.82	3.04	3.04	3.24	2.6	2.5
Arginine	4	3.97	3.95	3.91	3.90	4.02	4.03

[a] Moles of amino acid/mole of protein. Procedure for the calculation of the number of amino acids is the same as in the preceding paper (Fujioka and Scheraga, 1965). Correction factors for hydrolysis losses are: threonine, 1.05; serine, 1.12; half-cystine, 1.23; tyrosine, 1.14 (Gundlach *et al.*, 1959); and cysteic acid, 1.19 (Rupley and Scheraga, 1963). [b] Reoxidation after iodination of Ox-PIR. [c] A trace amount of glycine in the buffer solution was assumed not to have been removed completely by gel filtration. [d] Recoveries of tyrosine after hydrolysis of mono- and 3,5-diiodotyrosine were 92 and 76%, respectively. However, Cha and Scheraga (1963a) observed nearly complete recovery of tyrosine from their fraction B of iodinated ribonuclease A.

fractionated PIR (Figure 2) showed a single and almost symmetrical peak, indicating that this material was fairly homogeneous. This preparation was designated as sample A; most of the work in this paper was carried out on this sample. Chromatographic patterns of other preparations used in the present work were similar to that of sample A. Another preparation, sample B which will be mentioned below, was eluted slightly earlier than sample A.

Iodination products with less than 10 moles of I_2/mole of PIR or with 12 moles of I_2 and an insufficient reaction period (2 hours) showed two major peaks; no attempts were made to identify them.

Amino Acid Analyses of Iodinated PIR. Amino acid analyses of iodinated PIR and iodinated Ox-PIR together with Ox-PIR are listed in Table I. Since Cha and Scheraga (1963a) have reported that acid hydrolysis of the iodinated protein removes iodine from iodotyrosines to reproduce tyrosine, and that performic acid oxidation causes destruction of iodotyrosines to unidentified products but does not produce tyrosine, the analyses of the oxidized materials were carried out to determine the number of the uniodinated tyrosyl groups. In spite of the previous observation that PIR has only one abnormal tyrosine [as reported in the preceding paper (Fujioka and Scheraga, 1965)], the numbers of uniodinated tyrosines in iodinated PIR, as determined after oxidation, were always greater than 1 mole. As described below, the spectrophotometric titration data failed to provide a clear-cut resolution of this discrepancy. Nevertheless, since the number of uniodinated tyrosines is rather close to 1 mole, these samples (A and B) were used to locate the uniodinated tyrosine in the amino acid sequence of PIR.

It should be noted that about 1 mole of histidine was found to have been modified by iodination.[4] The numbers of the other amino acid residues were the same as those for PIR within experimental error.

Iodinated Ox-PIR (after reoxidation of the iodinated material) has been found to have a negligible amount of uniodinated tyrosine (about 0.2 mole/mole of protein)

[4] However, iodinated histidines could not be identified on the chromatogram.

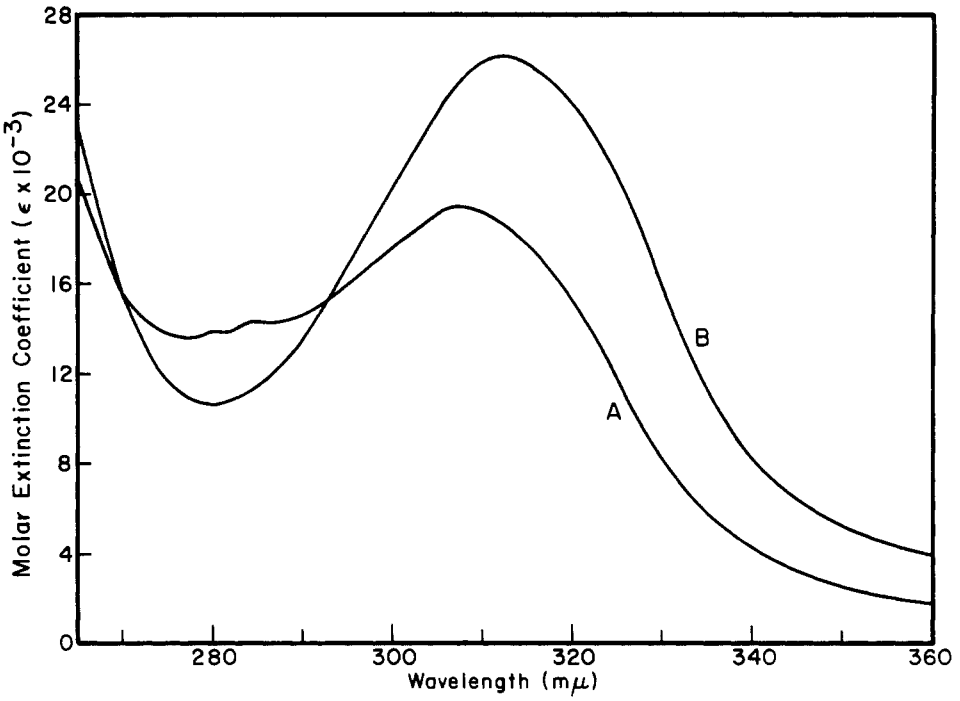

FIGURE 3: Ultraviolet absorption spectra at pH 10.5 (0.01 M borate buffer). Curve A, iodinated PIR (sample A); curve B, iodinated Ox-PIR.

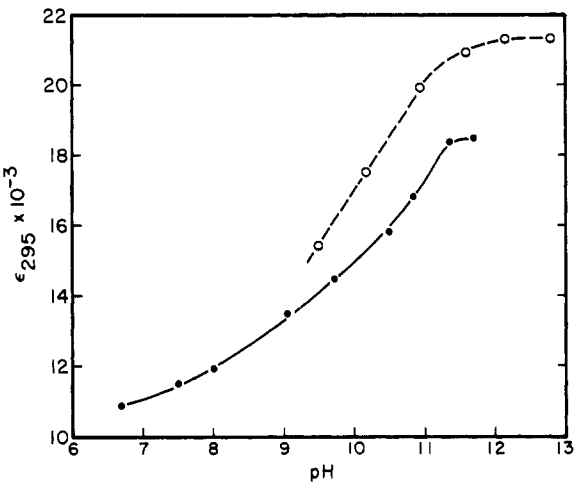

FIGURE 4: Spectrophotometric titration curves of iodinated PIR (sample A) at 295 mμ in 0.15 M KCl at 25°. ●, forward titration; ○, back titration after exposure for 1 hour to pH 12.8.

and an even smaller amount of histidine than iodinated PIR.

Spectral Studies. The ultraviolet absorption spectra at pH 10.5 of iodinated PIR (sample A) and iodinated Ox-PIR are shown in Figure 3. This pH value was chosen for comparison of these curves to those of iodinated ribonuclease A presented by Cha and Scheraga (1963a). Table II summarizes the molecular extinction coefficients (ϵ) and apparent ionization constants (pK_{app}) of iodotyrosines as measured in 0.15 M KCl at 25°, together with the reported values of ϵ and pK values observed in polypeptides. Iodinated Ox-PIR has its absorption maximum (λ_{max}) at 312 mμ which is identical with that of 3,5-diiodotyrosine (311 mμ), and all six normal tyrosines in Ox-PIR seem to have been converted to 3,5-diiodo derivatives. As for iodinated PIR, the general features of the curve are similar to those of iodinated ribonuclease A shown in Figure 4 of the paper by Cha and Scheraga (1963a). The observation that curve A of Figure 3 had its major peak at 308 mμ, *i.e.*, between 305 (λ_{max} of monoiodotyrosine) and 311 mμ (λ_{max} of 3,5-diiodotyrosine), suggested that it contained both kinds of iodotyrosines. The smaller peak near 285 mμ of sample A *might* indicate the presence of an uniodinated tyrosyl residue in the iodinated material. It may be concluded qualitatively that mono- and 3,5-diiodotyrosine, together with an abnormal tyrosine, are present in iodinated PIR. Quantitative spectral analyses of the iodinated protein, however, were difficult to make, as discussed by Cha and Scheraga (1963a).

Spectrophotometric titration experiments at 295 mμ were undertaken to determine the number of uniodinated tyrosyl residues in iodinated PIR. The titration curve of sample A is presented in Figure 4. Although iodinated PIR and iodinated Ox-PIR are insoluble below neutral pH, making it impossible to obtain the titration curve over the whole region of ionization of both iodotyrosines, an increase in optical density at 295 mμ between pH 7 and 12 may be at-

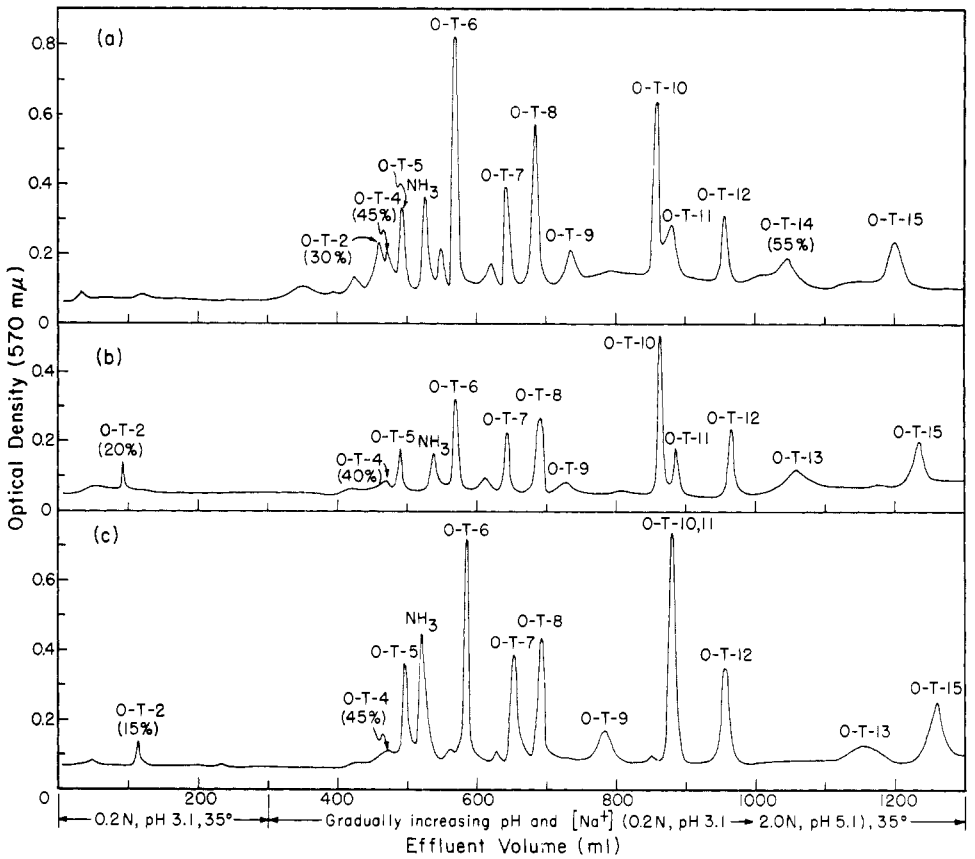

FIGURE 5: Chromatography of peptides from a 22-hour tryptic hydrolysate of oxidized protein on a 0.9 × 150 cm column of Dowex 50-X2. The effluent was collected in 2-ml fractions and the ninhydrin color value was obtained with the aid of a Technicon Autoanalyzer: (a) PIR, (b) sample A of iodinated PIR, (c) sample B of iodinated PIR. The percentages in parentheses represent the yields of the pertinent peptides.

TABLE II: Molar Extinction Coefficients and Apparent Ionization Constants of Phenolic Groups of Mono- and 3,5-Diiodotyrosine in 0.15 M KCl at 25°.[a]

	Low pH			High pH				
	λ_{max}, mμ	$\epsilon_{max} \times 10^{-3}$	$\epsilon_{295} \times 10^{-3}$	λ_{max}, mμ	$\epsilon_{max} \times 10^{-3}$	$\epsilon_{295} \times 10^{-3}$	pK_{app}	pK (in polypeptides)
Monoiodotyrosine	283	2.56	0.98	305	3.87	3.48	8.15	9.3[b]
	283	2.75[c]		305	3.85[c]			
3,5-Diiodotyrosine	285	2.58	2.41	311	5.83	3.64	6.38	7.5[b]
	285	2.73[d]		311	5.80[e]		6.5[e]	7.9[f]
				312	5.40[g]			7.7[h]

[a] The data taken from the references are those measured in water. [b] pK_{app} observed in thyroglobulin by Edelhoch (1962). [c] Herriott (1948). [d] Gemmill (1955). [e] Gruen *et al.* (1959). [f] pK_{app} observed in iodinated insulin by Gruen *et al.* (1959). [g] Katchalski and Sela (1953). [h] pK_{int} assumed for poly-3,5-diiodotyrosine by Katchalski and Sela (1953).

tributed to ionization of iodotyrosines; an increase in molar extinction coefficient ($\Delta\epsilon$) of 8300 observed for sample A (Figure 4) and 6000 for sample B (not shown) may be reasonably accounted for, if we assume that 2.5 moles each of mono- and 3,5-diiodotyrosine are present in iodinated PIR, since $\Delta\epsilon$ of 9300 [(2500 × 2.5) + (1210 × 2.5)] would be expected from the titration of iodotyrosines (Table II).

The irreversible ionization of uniodinated tyrosyl residues above pH 12 was observed as an increase in optical density at 295 mμ after exposure to high pH (see Figure 4). $\Delta\epsilon$ of about 2700 for sample A (Figure 4)

TABLE III: Amino Acid Compositions of Tyrosyl-Containing Peptides Isolated from Tryptic Digests of Oxidized Proteins.[a]

	O–T–2 (residues 67–85)				O–T–4 (residues 11–31)				O–T–14 (residues 92–98)		
			Iodinated PIR				Iodinated PIR				Iodinated PIR
Amino Acid	Theory	PIR[b]	Sample A	Sample B	Theory	PIR	Sample A	Sample B	Theory	PIR	Samples A and B
Cysteic acid	2	2.2	2.0	2.2	1	0.96	1.4	1.4	1	0.96	
Methionine sulfone	1	0.96	1.3	0.96	3	2.8	2.8	2.7			These peaks had disappeared
Aspartic acid	3	2.9	2.9	3.0	3	2.85	3.0	3.3	1	1.04	
Threonine	3	3.2	2.9	2.9	1	1.05	1.3	1.2			
Serine	3	3.0	2.8	3.3	6	6.3	6.1	6.1			No peptides were isolated
Glutamic acid	2	2.0	2.2	2.2	2	2.0	2.2	2.2		0.3	
Proline									1	0.94	
Glycine	1	1.0[c]	1.0[c]	1.0[c]			0.25	0.2			
Alanine		0.2	0.6		2	2.0[c]	2.0[c]	2.0[c]	1	1.0[c]	
Valine						0.2	0.25	0.2			
Isoleucine	1	1.1	0.76	0.88				0.2			
Tyrosine	2	1.9	0	0	1	0.90	1.0	0.84	2	1.95	
Lysine					1	0.97	1.15	1.15	1	0.92	
Histidine					1	1.05	0.90	0.91			
Arginine	1	1.1	0.90	0.92							
Yield of peptide, %		30	20	15		45	40	45		55	

[a] Moles of amino acid/mole of peptide. [b] The analysis was made on the front half of the peptide peak. [c] Assumed as reference.

and 2500 for sample B (not shown) were assigned to this increment in ϵ, although the end point of ionization of monoiodotyrosyl groups and the starting point of that of the abnormal tyrosyl group were not clearly separated. About 1 mole of abnormal tyrosine in iodinated PIR is thus suggested from the increment in ϵ at high pH. In this connection, $\Delta\epsilon$ of 2600/uniodinated tyrosyl residue has been found for PIR, as described in the preceding paper (Fujioka and Scheraga, 1965).

Tryptic Digestion of Oxidized Iodinated PIR. Figure 5 shows the peptide elution patterns of tryptic digests of oxidized PIR and oxidized iodinated PIR. The positions and yields of the peptides obtained from PIR (Figure 5a) were compared with those of previous reports (Hirs *et al.*, 1956a; Cha and Scheraga, 1963; Riehm *et al.*, 1965; Riehm and Scheraga, 1965). In general the yields were somewhat lower than those reported by Hirs *et al.* (1956a). The C-terminal peptide corresponding to O-T-16 from ribonuclease was not eluted with 2 N sodium acetate buffer, pH 6.6, at 50°.

Comparison of the peptide patterns from iodinated PIR (Figures 5b and 5c) with that from PIR (Figure 5a) indicated differences in the positions of some of the peptides. O-T-2 (residues 67–85) which contains tyrosine 73 and 76 was not found at its normal elution position. Instead, the peptide corresponding to O-T-2 was found at about 100-ml effluent volume and shown to contain no tyrosines (see Figures 5b and 5c and Table III). O-T-14 (residues 92–98) which has tyrosines 92 and 97 disappeared, but in this case the corresponding peptide without tyrosines could not be detected on the chromatogram. The reason why O-T-13, which consists of residues 38–61 (*i.e.*, the Arg 39–Cys 40 bond was not quantitatively cleaved), was left in the digests from iodinated PIR was not clear, especially since observable amounts of O-T-7 (residues 38 and 39) and O-T-9 (residues 40–61) did appear. Analyses of these peaks did not provide any evidence for the presence of O-T-14.

On the other hand, O-T-4 which contains tyrosine 25 was found in the digests of both PIR and iodinated PIR. All the peptides except O-T-2, O-T-13, and O-T-14 were obtained in comparable yields for both proteins.

As pointed out by Riehm *et al.* (1965), the region between O-T-2 and O-T-4 was suspected to be contaminated by other peptides due to the presence of a small amount of chymotrypsin in the trypsin preparation; special care was therefore taken in the analyses of these two peaks. The front half of the peak was analyzed for O-T-2 as recommended by Riehm *et al.* (1965). In

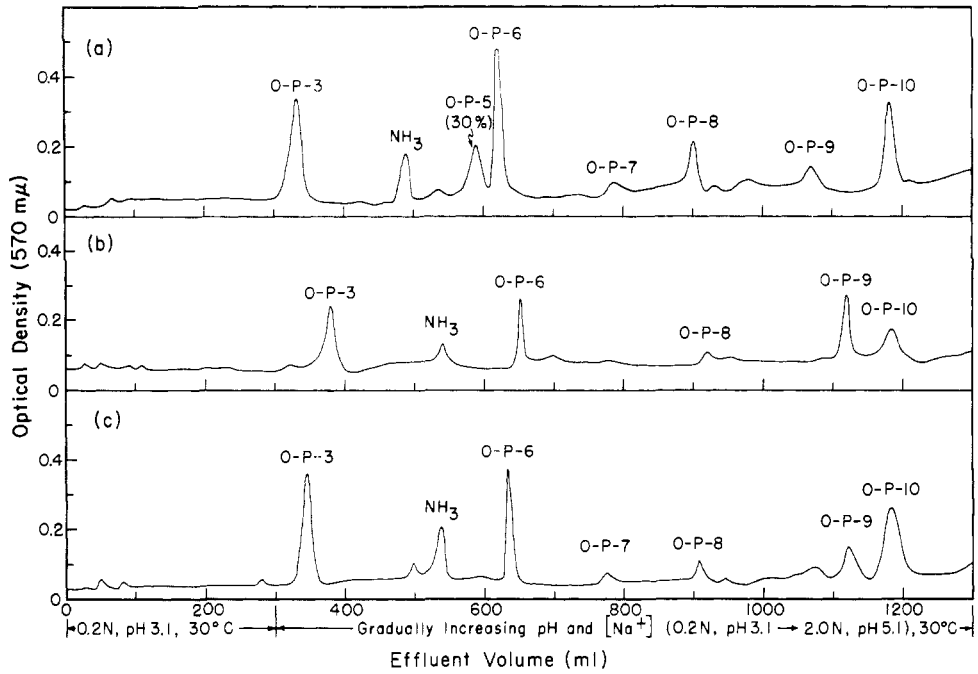

FIGURE 6: Chromatography of peptides from a 22-hour peptic hydrolysate of oxidized protein on a 0.9 × 150 cm column of Dowex 50-X2. The analytical procedures were the same as for the tryptic digests (Figure 5): (a) PIR; (b) sample A of iodinated PIR; (c) sample B of iodinated PIR. The percentage in parenthesis represents the yield of O-P-5 from PIR.

the present study, O-T-2-C-5 (a chymotryptic digest of O-T-2; Hirs, 1960) was not found in peak O-T-4; thus, the whole peak of O-T-4 was used for analysis. The amino acid analyses and the yields of tyrosine-containing peptides (O-T-2, O-T-4, and O-T-14) are compared in Table III.

From reoxidized iodinated Ox-PIR (not shown), neither O-T-4 nor O-T-14 could be identified, while the peptide corresponding to O-T-2 which had no tyrosines was eluted at the front of the chromatogram as in the case of iodinated PIR (see Figure 5b and 5c).

Peptic Digestions. Peptic hydrolyses were carried out to isolate the C-terminal peptide of PIR which contains tyrosine 115. Chromatographic patterns of the digests from PIR and iodinated PIR are presented in Figure 6. The elution pattern from PIR (Figure 6a) is similar to those reported by Bailey *et al.* (1956), Cha and Scheraga (1963), and Riehm and Scheraga (1965), except that O-P-2 which is the C-terminal tetrapeptide is missing in PIR. Comparison of Figure 6a with Figure 6b and 6c, the elution patterns from iodinated PIR, indicates that O-P-5 (residues 109–120) has disappeared in the digests from iodinated PIR.[5] The remaining peaks from PIR were shown not to contain tyrosyl residues. Those peptides from iodinated PIR showed similar patterns and yields. The amino acid composition of O-P-5 from PIR is presented in Table IV.

TABLE IV: Amino Acid Composition of O–P–5 (Residues 109–120).[a]

Amino Acid	Theory	PIR	Iodinated PIR Samples A and B
Cysteic acid	1	1.0	
Aspartic acid	1	1.1	
Threonine		0.21	These peaks had
Serine		0.22	disappeared
Glutamic acid	1	1.0	No peptides
Proline	2	2.4	were isolated
Glycine	1	1.0[b]	
Alanine	1	0.96	
Valine	2	1.9	
Tyrosine	1	1.0	
Phenylalanine	1	0.99	
Histidine	1	0.90	
Yield of peptide, %		30	

[a] Moles of amino acid/mole of peptide. [b] Assumed as reference.

Chymotryptic Digestions. It was demonstrated by tryptic digestion that tyrosine 25 had not been iodinated, whereas tyrosines 73 and 76 had been iodinated. Chromatographic analyses of chymotryptic digests of the oxidized proteins were attempted to determine the

[5] The disappearance of these peptides will be discussed later.

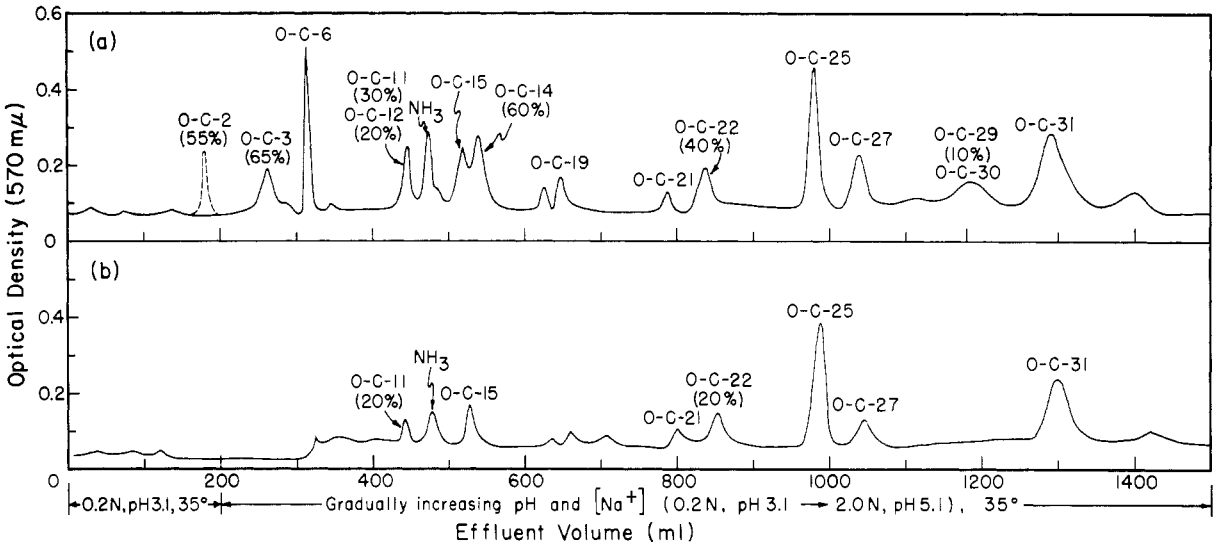

FIGURE 7: Chromatography of peptides from a 22-hour chymotryptic hydrolysate of oxidized protein on a 0.9 × 150 cm column of Dowex 50-X2. The analytical procedures were the same as for the tryptic and peptic digests (Figures 5 and 6). The ninhydrin-negative peptide O-C-2 was detected after alkaline hydrolysis of tubes 50–110 and shown by a dotted line: (a) PIR, (b) sample A of iodinated PIR. The percentages in parentheses represent the yields of the pertinent peptides.

TABLE V: Amino Acid Compositions of Peptides Isolated from Chymotryptic Digests of Oxidized Proteins.[a]

	O–C–2 (residues 74–76)			O–C–3 (residues 106–115)			O–C–14 (residues 80–97)		
Amino Acid	Theory	PIR	Iodinated PIR Sample A	Theory	PIR	Iodinated PIR Sample A	Theory	PIR	Iodinated PIR Sample A
Cysteic acid				1	1.45		2	2.2	
Aspartic acid			This peak had	1	1.5	This peak had	2	2.1	This peak had
Threonine			disappeared		0.4	disappeared	2	2.0	disappeared
Serine	1	0.95					3	3.0	
Glutamic acid	1	1.0[b]		1	1.1		1	1.0	
Proline				1	0.70		1	1.0	
Glycine				1	0.97		1	1.0	
Alanine				1	1.0[b]		1	1.0[b]	
Valine				1	0.99				
Isoleucine				2	1.2		1	0.95	
Tyrosine	1	0.96		1	0.94		2	2.0	
Phenylalanine									
Lysine							1	1.1	
Arginine							1	1.1	
Yield of peptide, %		55			65			60	

[a] Moles of amino acid/mole of peptide. [b] Assumed as reference.

state of iodination of the other three tyrosines. The elution pattern from PIR (Figure 7a) was similar to those reported by Hirs *et al.* (1956b), Cha and Scheraga (1963b), and Riehm *et al.* (1965). The ninhydrin-negative peptide O-C-2 was detected after alkaline hydrolysis and indicated by a dotted line in Figure 7a. Major differences from previous studies are (1) O-C-15 (residues 47–62) and O-C-14 (residues 80–97, containing tyrosines 92 and 97) were eluted in two separate peaks in this order, and (2) O-C-4 which is the

TABLE VI: Amino Acid Analyses of Peptide Mixture of O–C–11 (Residues 19–25) and O–C–12 (Residues 47–73) Obtained from Chymotryptic Digests.

Amino Acid	Theory O–C–11	Theory O–C–12	PIR Exptl, μmole	Ratio of Exptl to Calcd Values[a]	Iodinated PIR[b] O–C–11	O–C–12 (Sample A)
Cysteic acid		3	0.356	0.99		
Aspartic acid	1	4	0.666	1.0	1.15	This peak had
Threonine		1	0.109	0.9		disappeared
Serine	3	2	0.795	1.0	2.95	
Glutamic acid		4	0.510	1.1		
Glycine		1	0.146	1.2		
Alanine	2	3	0.715	0.99	2.0[c]	
Valine		4	0.426	0.89		
Leucine		1	0.121	1.0		
Tyrosine	1	1	0.282	0.94	0.90	
Lysine		2	0.277	1.15		
Histidine		1	0.11	0.92		
Yield of peptide, %			O–C–11, 30 O–C–12, 20		20	

[a] For 0.18 μmole of O-C-11 and 0.12 μmole of O–C–12. [b] Moles of amino acid/mole of peptide. [c] Assumed as reference.

C-terminal tetrapeptide was missing. The yields of the peptides were generally somewhat lower than those reported by Hirs *et al.* (1956b).

The chromatographic pattern from sample A of iodinated PIR is shown in Figure 7b. Alkaline hydrolysis was also carried out to detect the ninhydrin-negative O-C-2. Comparison of the elution curves of Figure 7a and 7b indicates distinct differences in that peaks O-C-2 (residues 74–76), O-C-3 (residues 106–115), O-C-6 (residues 77–79), O-C-14 (residues 80–97), O-C-19 (residues 98–103), and a mixture of O-C-29 (residues 104–115) and O-C-30 (residues 116–120) disappeared from iodinated PIR. The peak which is labeled as O-C-11 in Figure 7b was shown not to contain O-C-12 (residues 47–73) as explained below. Among these peptides only O-C-2, O-C-12, O-C-14, O-C-3,[5] and O-C-29[5] contain tyrosyl residues. The disappearance of the peptides which do not contain tyrosine, O-C-6, O-C-19, and O-C-30,[5] may be interpreted as arising from the fact that chymotrypsin failed to hydrolyze the peptide bonds adjacent to the modified tyrosyl groups. The amino acid compositions of O-C-2, O-C-3, and O-C-14 from PIR are presented in Table V.

The amino acid analysis of the mixture of O-C-11 and O-C-12 from PIR was interpreted by calculation of the data as follows. Peptide O-C-12 has several amino acids which do not occur in peptide O-C-11: thus the amount of O-C-12 was calculated as 0.12 μmole on the basis of the leucine value. The amount of O-C-11 was then calculated on the basis of the value of aspartic acid, *i.e.*, 0.18 μmole. The fifth column of Table VI presents the ratio of experimental values (the fourth column) to calculated values. The amino acid analysis of the peptide in the region of the peak labeled O-C-11 (Figure 7b) showed that only O-C-11 (residues 19–25) was eluted in the digest of oxidized iodinated PIR, while this peptide was mixed with O-C-12 in the digest from PIR (Figure 7a). The result of the analysis of the mixture of O-C-11 and O-C-12 from PIR together with the amino acid composition of O-C-11 obtained from sample A of iodinated PIR are presented in Table VI. A similar calculation was carried out for the analysis of the mixture O-C-29 and O-C-30 obtained from PIR (Table VII).

Another tyrosine-containing peptide, O-C-22 (residues 9–25), was isolated from both PIR and iodinated PIR (Figure 7a and 7b). The amino acid analyses of these peaks are presented in Table VIII. It should be noted that not only O-C-22 but also O-C-27 (residues 26–35) was recovered from iodinated PIR (Figure 7b); this observation would emphasize the presence of the uniodinated tyrosyl residue at position 25.

Discussion

In the present study, the location of the abnormal or "buried" tyrosyl group in the amino acid sequence of PIR was attempted. It was desirable to obtain a derivative in which all the normal or "exposed" tyrosines are modified without any conformational change in the protein. Iodination of the protein at pH 9.5 and 0° (Hughes and Straessle, 1950) seemed fairly ideal for this purpose. Indeed, Cha and Scheraga (1963a,b) have succeeded in the identification of two of the three ab-

TABLE VII: Amino Acid Analyses of Peptide Mixture of O–C–29 (Residues 104–115) and O–C–30 (Residues 116–120) Obtained from Chymotryptic Digests.

Amino Acid	Theory O–C–29	Theory O–C–30	PIR Exptl, μmole	Ratio of Exptl to Calcd Values[a]	Iodinated PIR (Sample A) O–C–29 / O–C–30
Cysteic acid	1		0.07	1.1	
Aspartic acid	1		0.065	1.0	These peaks had
Glutamic acid	1		0.068	1.05	disappeared
Proline	1	1	0.203	0.88	
Glycine	1		0.066	1.0	
Alanine	1		0.065	1.0	
Valine	1	2	0.332	0.84	
Isoleucine	2		0.07	1.1	
Tyrosine	1		0.07	1.1	
Phenylalanine		1	0.165	1.0	
Lysine	1		0.07	1.1	
Histidine	1	1	0.159	0.98	
Yield of peptide, %			O–C–29, 10 O–C–30, 30		

[a] For 0.165 μmole of O–C–29 and 0.065 μmole of O–C–30.

TABLE VIII: Amino Acid Composition of O–C–22 (Residues 9–25.)[a]

Amino Acid	Theory	PIR	Iodinated PIR (Sample A)
Methionine sulfone	1	0.95	1.1
Aspartic acid	2	2.0	2.1
Threonine	1	0.85	1.0
Serine	6	6.0	6.0
Glutamic acid	2	1.9	2.0
Alanine	2	1.9	1.8
Tyrosine	1	1.0	0.98
Histidine	1	0.97	0.93
Arginine	1	1.0[b]	1.0[b]
Yield of peptide, %		40	20

[a] Moles of amino acid/mole of peptide. [b] Assumed as reference.

normal tyrosines in ribonuclease (Shugar, 1952; Tanford *et al.*, 1955) by this procedure. Although there was no evidence that indicated that the native structure was maintained in the iodinated derivative, it was assumed that the uniodinated and abnormal tyrosine in iodinated PIR was still involved in the same interaction with the other group (possibly a "buried" carboxyl group) in the protein. Because of the insolubility of iodinated PIR below neutral pH, titration of the carboxyl groups or a difference spectrum measurement at low pH could not be carried out.

In contrast to previous work on iodination of ribonuclease A (Cha and Scheraga, 1963a), the iodinated derivatives of PIR seemed fairly homogeneous. It might be due to the less rigid structure of PIR (Sela and Anfinsen, 1957; Sela *et al.*, 1957; Ottesen and Stracher, 1960), *i.e.*, the normal tyrosines might be fairly uniformly iodinated in the presence of excess iodine (12 moles of I_2/mole of PIR), and with the reaction period of 4 hours. Since a spectral study indicated that all six tyrosyl groups in Ox-PIR seemed to have been modified to 3,5-diiodo derivatives (Figure 3, curve B), the probable presence of monoiodotyrosine in iodinated PIR as revealed by its absorption spectrum (Figure 3, curve A) suggested some conformational restriction by the environments of tyrosyl groups in this protein. Similar observations have been reported for the iodinated derivatives of ribonuclease A (Cha and Scheraga, 1963a).

The major point of interest was the number of uniodinated tyrosyl groups in the iodinated protein. The determination of free tyrosine after oxidation of iodinated protein, which is considered the most reliable method at the moment, always gave more than 1 mole of tyrosine in iodinated PIR (Table I). Spectrophotometric titration at 295 mμ of iodinated PIR did not provide a clear-cut resolution of this problem because of a rather large increase in optical density at 295 mμ below pH 12 due to ionization of iodotyrosyl groups (Figure 4). Furthermore, the ionization of unreacted normal tyrosine, if any, could overlap in this region. The possibility is thus suggested of incom-

plete reaction of normal tyrosyl groups in PIR with iodine and inhomogeneity of the iodinated derivative. However, neither prolongation of the reaction (up to 8 hours) nor modification of chromatographic elution conditions of the iodinated products was successful.

Another difficulty in the present work was the modification of about 1 mole of histidine upon iodination of PIR as revealed by amino acid analysis data (Table I). Since Cha and Scheraga (1963a) did not find any change in histidine content even in the exhaustively iodinated derivative of ribonuclease A (their fraction E), there might be some difference in reactivity of histidine residue(s) toward iodine between the two proteins. Modification of the amino acids other than tyrosine and histidine by iodine could not be detected by amino acid analyses (Table I).

In spite of the difficulties described above, it was decided to study the peptides obtained from proteolytic digests of oxidized iodinated derivatives in order to identify the "uniodinated" tyrosine in the amino acid sequence in PIR. The principle of this work, as has been discussed before by Cha and Scheraga (1963b), is based on the assumption that the yields and compositions of peptides containing free tyrosine from iodinated PIR should be comparable to those of the corresponding peptides from PIR. Modification of tyrosyl groups should result in disappearance of the peptides or their shift to other positions in the chromatogram. Thus, from the tryptic digestion experiments, it was shown that tyrosines 73 and 76 had been iodinated, while tyrosine 25 was intact. O-T-2 containing tyrosines 73 and 76 was not found in its normal position and the corresponding peptide missing tyrosines was detected in a different position from iodinated PIR. O-T-4 containing tyrosine 25 was found in comparable yields from both the iodinated and uniodinated protein. The disappearance of O-T-14 from iodinated PIR indicated the modification of tyrosine 92 and/or 97. As to peptic digestions, O-P-5 containing tyrosine 115 and histidine 119 disappeared from iodinated PIR, showing that this tyrosine and/or histidine had been modified. Further evidence that tyrosines 73 and 76 had been iodinated was obtained by chymotryptic digestions: both O-C-12 and O-C-2 disappeared. The identification of the state of iodination of tyrosine 115 was difficult to determine because of the possible accompanying iodination of histidine 105 or 119. The isolation of O-C-11 and O-C-22, both of which contain tyrosine 25, from iodinated PIR supported the result of the tryptic digestion that tyrosine 25 had not been iodinated, although the yields of these peptides were lower than those from PIR. Finally, it was confirmed that tyrosine 97 had been iodinated since both O-C-14 (residues 80–97) and O-C-19 (residues 98–103) were not found in the digest from iodinated PIR. The state of tyrosine 92 could not be determined from these peptide analyses.

Another problem was the location of the modified histidine. Since the peptides containing histidine 12 or 48 were found from iodinated PIR to contain about 1 mole each of histidine, it was concluded that these residues had not been modified. These peptides are O-T-4 (Table III), O-C-21 (residues 9–18), O-C-22 (Table VIII), O-C-12 (Table VI), O-C-15 (residues 47–62), and O-P-10 (residues 46–51). The disappearance of peptides O-C-29, O-C-30, and O-P-5 from iodinated PIR could not lead to any definite choice between histidine 105 and 119 as the modified residue. However, in view of the observation that the C terminus of PIR is exposed, as reported in the preceding paper (Fujioka and Scheraga, 1965), it is more probable that histidine 119 had been iodinated.

Although there remains ambiguity concerning tyrosines 92 and 115, it was concluded that the uniodinated tyrosine in iodinated PIR is tyrosine 25, since iodinated PIR has only one abnormal tyrosine and tyrosine 25 was shown to be present as the free residue in oxidized iodinated PIR. In support of this, peptide O-T-4 disappeared from reoxidized iodinated Ox-PIR, which was believed to contain no free tyrosine. Although we have no evidence that no conformation change occurred near the tyrosyl residues during the iodination reaction, the evidence suggests that tyrosine 25 is the single buried tyrosyl residue in PIR.

As noted in the preceding paper, PIR-2, which has inner cleavages at the bonds Met-Ser (79-80) (and, to a lesser degree, Thr-Phe (45-46) and Glu(NH_2)-Ala (55-56)), contains one abnormal tyrosyl group. Since PIR-2 preparations were inhomogeneous and a sufficient amount of material was not available, an iodination experiment could not be carried out. It may be reasonably inferred, however, that the molecule having the bond Met-Ser (79-80) split still contains the abnormal tyrosine, presumably in position 25.

Recently a three-dimensional structure of ribonuclease has been proposed by Saroff (1965) based on ion-binding studies of this protein (Saroff and Carroll, 1962; Loeb and Saroff, 1964). According to his model the abnormal tyrosine in PIR was predicted at tyrosine 25, quite consistent with the present result.

Two of the buried tyrosyl groups in ribonuclease A have been identified as tyrosines 25 and 97 (Cha and Scheraga, 1963b), and the three buried carboxyl groups as aspartic acids 14, 38, and 83 (Riehm *et al.*, 1965). The choice of pairing the "buried" tyrosyl and carboxyl groups has been tentatively made as tyrosine 25 to aspartic acid 83 and tyrosine 97 to aspartic acid 38 (Riehm *et al.*, 1965). In the present study it has been suggested that tyrosine 25 is the "buried" one in PIR. Attempts to identify the "buried" carboxyl group(s) in PIR are now in progress. The results of these experiments will provide further information about the proper pairing of the tyrosyl and carboxyl groups in ribonuclease A.

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