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Structural Studies of Ribonuclease. XXVI. The Role of Tyrosine 115 in the Refolding of Ribonuclease*

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ABSTRACT: Iodination of the tyrosine residues of bovine pancreatic ribonuclease has yielded a number of enzymically active derivatives which were separated, purified, and characterized. They differ with respect to the number of tyrosine residues iodinated and with respect to the number of iodine atoms per tyrosine. The reversibility of reduction and denaturation of these derivatives was used as a test of whether they still possessed all of the information required for the attainment of the enzymically active three-dimensional conformation.

Reversibility was maintained only in those derivatives in which tyrosine residue 115 had not been diiodinated, and was in the protonated form. We have concluded that this residue, in its protonated form, contributes a required piece of information to the polypeptide chain in the process by which renaturation occurs.

The reversible denaturation of bovine pancreatic ribonuclease (RNAase) by disulfide bond cleavage was first demonstrated by Sela *et al.* (1959). After reductive cleavage of the disulfide bonds and disruption of its three-dimensional structure in 8 M urea, the protein was found to be devoid of enzymic activity. However, after removal of these reagents, and under appropriate conditions, the polypeptide chain spontaneously underwent oxidation, with correct pairing of half-cystine residues, and refolded in such a way as to reestablish the active three-dimensional conformation characteristic of the native protein. This observation led to the conclusion that all the information required for the attainment of the native three-dimensional conformation of a protein resides within the amino acid sequence of the polypeptide chain (Anfinsen, 1962).

In an effort to discover what interactions play a role in governing the refolding process, the reversibility was studied in a number of enzymically active derivatives of RNAase which had been prepared chemically (Anfinsen *et al.*, 1962; Epstein and Goldberger, 1964). The rationale for experiments of this type was that, if well-defined chemical modifications prevented the polypeptide chain from refolding properly, one might be able to infer part of the "code" by which linear amino acid sequences dictate specific three-dimensional conformations. However, all of the derivatives examined were capable of regaining the active conformation after denaturation, and therefore only negative conclusions could be drawn concerning the question of what are essential items of information in guiding the refolding process.

During the course of studies of the iodination of RNAase (Woody *et al.*, 1966), it was found that the enzymic activity of a partially iodinated derivative could *not* be restored after reduction and reoxidation. This paper describes the preparation of several derivatives of ribonuclease, in each of which the number of iodinated tyrosyl residues differs. From a study of the reversible reduction and reoxidation of the disulfide bonds in each of these derivatives it appears that tyrosine 115 is involved in directing the refolding of the molecule to form the enzymically active structure.

Experimental Section

Materials. Bovine pancreatic RNAase, five times recrystallized, was purchased from Sigma Chemical Co. (lot no. 65B-0350). It was further purified by chromatography on a 7.5 × 60 cm column of unsieved

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Amberlite IRC-50,XE-64 in 0.2 M sodium phosphate buffer, pH 6.47 (Hirs *et al.*, 1953). The fraction containing RNAase-A was deionized by passage through a 3.3 × 35 cm column of MB-1, mixed-bed resin (Rohm and Haas), lyophilized, and stored at 4° until needed.

Crystalline trypsin (twice recrystallized, lot 6108) and salt-free chymotrypsin (lot CD1-678-8413) were purchased from the Worthington Biochemical Corp., and were used without further purification. Yeast ribonucleic acid (RNA) was purchased from Sigma Chemical Co. Urea (Mallinckrodt Chemical Works) was recrystallized from a 70% ethanol–water solution. β-Mercaptoethanol was purchased from City Chemical Corp., formic acid (99% pure) from Matheson Coleman and Bell, and 30% hydrogen peroxide from Merck and Co.

Sephadex G-25 and superfine G-25 were purchased from Pharmacia of Sweden. Analytical grade Dowex 50-X2 was purchased from Bio-Rad Laboratories, and it was prepared according to the recommendations of Hirs *et al.* (1956a). Carboxymethylcellulose (CM 11) was obtained from the Whatman Co.

The iodinating reagent, which was 0.05 M in I_2 and 0.2 M in KI, was standardized against As_2O_3 obtained from Mallinckrodt Chemical Works. Constant-boiling HCl was used in the acid hydrolysis of peptides and proteins. All other chemicals were reagent grade unless otherwise specified.

Iodination of RNAase. The Iodination was carried out according to the method outlined by Woody *et al.* (1966). When 4 moles of iodine/mole of RNAase (I_4 RNAase) was added the mixture was allowed to stand at 4° for 4 hr before dialysis. When 2 moles of iodine/mole of RNAase (I_2 RNAase) was used the mixture was allowed to stand for 30 min at 4° before dialysis. After dialysis, the solutions were lyophilized. The preparation of a derivative (I_6 RNAase) by addition of 6 moles of iodine/mole of RNAase (and its fractionation and characterization) was described previously (Woody *et al.*, 1966).

Fractionation of Iodinated RNAase. Fractionation of the iodinated derivatives by chromatography on carboxymethylcellulose (Taborsky, 1959) was carried out as described by Woody *et al.* (1966). The fractions were desalted as described by the latter workers.

Spectral Measurements. The molar extinction coefficients of the iodinated derivatives at 278 mμ were determined according to the method of Woody *et al.* (1966). The molar extinction coefficient of both fractions B and C from I_4 RNAase (see below) was 12,070 ± 200. The molar extinction coefficient of fraction C from I_2 RNAase (see below) was 10,920 ± 200. The value for RNAase is 9800 (Hermans and Scheraga, 1961).

Ultraviolet difference spectra at pH 11.2 or 11.3 *vs.* 2.1 of fractions B and C from I_4 RNAase and of fraction C from I_2 RNAase were measured at 25° with a Cary Model 14 recording spectrophotometer using quartz cells with 10-mm light path. These pH values were selected because all the exposed tyrosine residues (iodinated and uniodinated) are ionized at the high pH and un-ionized at the low pH; also, the buried tyrosine residues remain un-ionized at these pH values. Variation of the reference pH from 2.1 to 4.5 did not alter the appearance of the difference spectra.

Performic Acid Oxidation. Performic acid oxidation was carried out according to the method of Hirs (1956). After the samples had been oxidized and lyophilized they were passed through a Sephadex column (previously equilibrated with 1 M acetic acid) to remove any iodine that may have been liberated.

Enzymic Digestion. Tryptic and chymotryptic digestions (Hirs *et al.*, 1956a,b) were carried out according to the procedures described by Cha and Scheraga (1963). Between 30 and 40 mg of oxidized protein was used.

Amino Acid Analyses. These analyses (Gundlach *et al.*, 1959) were performed with a Technicon Autoanalyzer according to the recommendations of Cha and Scheraga (1963). Samples containing large quantities of salt were desalted after hydrolysis according to the method of Woody *et al.* (1966). Correction factors for destruction of cysteic acid, threonine, serine, and tyrosine were also applied (Rupley and Scheraga, 1963).

By assuming a theoretical number for the amount of one amino acid, the amounts of the other residues could be calculated by comparison with this theoretical value. The dry weight of the oxidized protein was obtained by making a correction for 10% moisture.

Reduction and Reoxidation of RNAase and Its Iodinated Derivatives. The reduction and reoxidation of RNAase and its iodinated derivatives were carried out according to the method of Anfinsen and Haber (1961). The reduction was carried out as follows. In a 10-ml erlenmeyer flask 10 mg of dry protein was dissolved in 1.0 ml of a solution which was 8 M in urea, 0.3 M in β-mercaptoethanol, and 0.06 M in Tris–acetate. The final pH of this solution was 8.2. The flask was tightly stoppered and incubated at 25° for 16 hr. The solution of reduced protein was then subjected to gel filtration on a 3.5 × 30 cm column of superfine Sephadex G-25 which had previously been equilibrated with 0.1 M acetic acid. The flow rate was about 40 ml/hr. Fractions of 5 ml were collected and the optical density was measured at 278 mμ. The protein fractions were pooled and the optical density was again measured at 278 mμ. A diminution of 3.5% in the extinction coefficient of reduced RNAase in comparison to that of the native material was observed by White (1961). The same per cent diminution was assumed for the reduced iodinated derivatives. Using the extinction coefficient of the reduced material, a 0.2-mg/ml solution was prepared and stored at 4°. At low pH the reduced protein will not reoxidize (Anfinsen and Haber, 1961). The material was freshly prepared every few days.

Reoxidation was carried out both in polyethylene and in siliconized glass vessels. Nonsiliconized glass tends to denature dilute solutions of RNAase (Shapira, 1959). One milliliter of the reduced protein solution (0.2 mg/ml) was added to 9 ml of 0.1 M Tris buffer, pH 8.6,

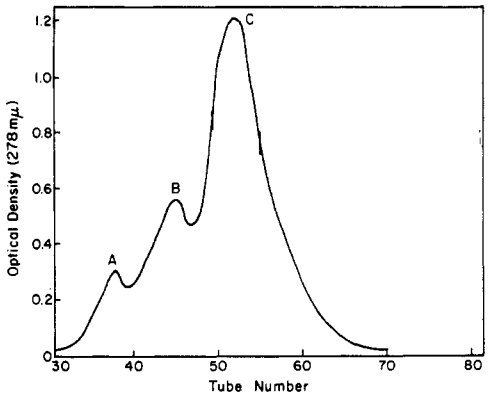

FIGURE 1: Chromatography of I_2 RNAase (300–400 mg) on a 2.5 × 40 cm column of carboxymethylcellulose. A two-stage linear gradient was employed. The first stage contained 1.2 l. of 0.005 M Tris buffer + 0.001 M EDTA, pH 8.5; the second stage contained 1.2 l. of 0.005 M Tris buffer + 0.001 M EDTA + 0.15 M sodium acetate, pH 8.5. The flow rate was 60 ml/hr, and the effluent was collected in 10-ml fractions. The optical density was read at 278 mμ. Fraction C was pooled separately as indicated.

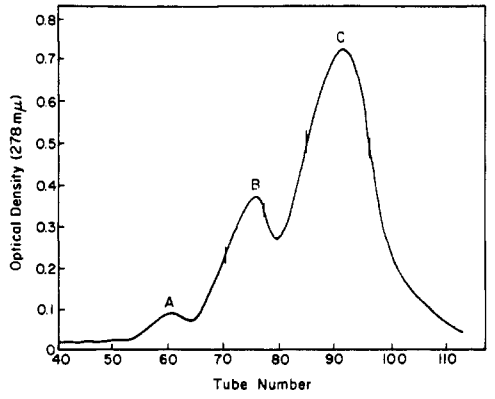

FIGURE 2: Chromatography of I_4 RNAase (300–400 mg) on a 2.5 × 40 cm column of carboxymethylcellulose. The gradient used was the same as stated in the legend for Figure 1. Fractions B and C were pooled separately as indicated.

in a 125-ml siliconized or polyethylene erlenmeyer flask. The mixture was allowed to stand at 25°, and activity measurements were made the following day on the reoxidized, reduced, and native material.

Enzyme Assay. The activity measurements were carried out according to the method of Anfinsen *et al.* (1954). Aliquots (0–50 μl) of the protein solution (containing 0–1 μg of protein) were diluted to 1.5 ml with 0.1 M sodium acetate buffer, pH 5. A 0.02-mg/ml solution of the inactive reduced protein, previously stored at 4°, was also prepared for the activity assay. RNA (1 ml of 0.4%) (Crestfield *et al.*, 1956) was added

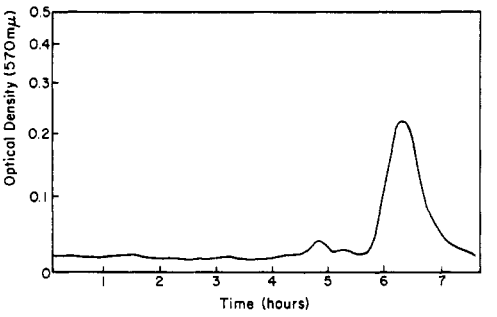

FIGURE 3: Analytical rechromatography of component I_2 RNAase-C on a 0.9 × 30 cm column of carboxymethylcellulose. A two-stage gradient was employed. The first stage contained 100 ml of 0.005 M Tris buffer containing 0.001 M EDTA, pH 8.5; the second stage contained 100 ml of 0.005 M Tris containing 0.001 M EDTA and 0.15 M sodium acetate, pH 8.5. The ninhydrin color value of the effluent (flow rate of 20 ml/hr) was obtained with the acid of a Technicon Autoanalyzer.

to these solutions, and the reaction was allowed to proceed for 25 min at 25°. At the end of this period the reaction was stopped by the addition of 0.5 ml of a uranyl acetate solution (prepared by dissolving 7.5 g of uranyl acetate in 1 l. of 25% perchloric acid). High molecular weight (undigested) RNA and RNAase were thus precipitated, leaving the low molecular weight nucleotides in solution. The precipitate was removed by centrifugation, and 0.1 ml of the supernatant liquid was added to 3 ml of distilled water. The optical density of this solution was measured at 260 mμ. Control assays, from which protein had been omitted, were carried through the experiment.

Results

Fractionation of Iodinated Derivatives. The results obtained in the fractionation of the iodinated derivatives are shown in Figures 1 and 2. The relative yield of each fraction from I_2 RNAase was estimated from the areas under the curves shown as 10% A, 19% B, and 71% C (Figure 1). Fraction C was collected, pooled as indicated in Figure 1, and designated "I_2 RNAase-C." The relative yield of each fraction from I_4 RNAase was estimated as 8% A, 25% B, and 67% C (Figure 2). Fractions B and C were collected, pooled as indicated in Figure 2, and designated "I_4 RNAase-B" and "I_4 RNAase-C," respectively. All fractions were then purified by passage through a 2.5 × 50 cm column of Sephadex G-25 equilibrated with 0.05 M ammonium acetate, exhaustively dialyzed against distilled water, and lyophilized. Approximately 5 mg of each of the three samples was analyzed on an analytical column, and the results are shown in Figures 3 and 4; the samples were considered to be fairly homogeneous, and were used without further purification.

Ultraviolet Difference Spectra. I_2 RNAase-C. The ultraviolet difference spectrum of I_2 RNAase-C is

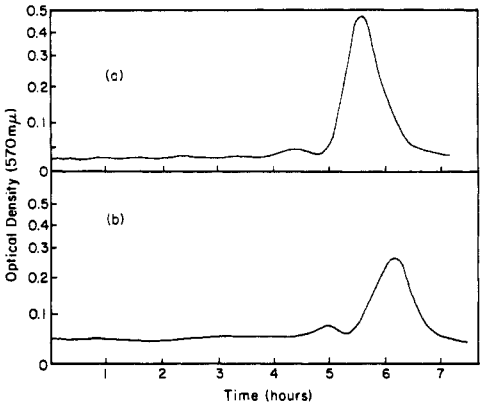

FIGURE 4: Analytical rechromatography of components I_4 RNAase-B and I_4 RNAase-C, in a and b, respectively. The gradients used were the same as stated in the legend for Figure 3.

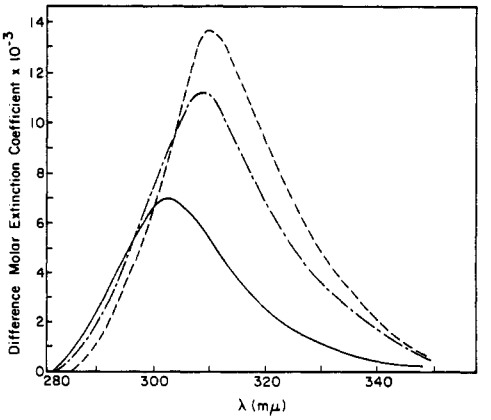

FIGURE 5: Ultraviolet difference spectra of I_2 RNAase-C (———), I_4 RNAase-B (– – – –), and I_4 RNAase-C (–·–·–·–). The reference solutions were at pH 2.1, 25°, whereas the test solutions were at pH 11.3 (except for I_2 RNAase-C, which was at pH 11.2), 25°.

shown in Figure 5. The values of λ_{max} and $\Delta\epsilon_{max}$, obtained from this curve, are shown in Table I, together with the corresponding values of monoiodotyrosine and diiodotyrosine which were previously reported by Woody *et al.* (1966). Since λ_{max} for I_2 RNAase-C is 303 mμ, it appears that the iodinated tyrosyl group(s) of I_2 RNAase-C is (are) in the monoiodinated form. The observed value of 7000 for $\Delta\epsilon_{max}$, compared to 3600 for monoiodotyrosine, indicates that I_2 RNAase-C contains *two* monoiodotyrosyl groups. The data for I_2 RNAase-C are incompatible with the interpretation that either or both of the iodinated tyrosyl residues is in the diiodinated form.

TABLE I: Maximum Wavelengths (λ_{max}) and Difference Molar Extinction Coefficients ($\Delta\epsilon_{max}$) of Monoiodotyrosine, Diiodotyrosine, Mixtures of Monoiodo- and Diiodotyrosine, and Iodinated RNAase Derivatives from the Ultraviolet Difference Spectra at pH 11.2–11.4 *vs.* 2.1 (at 25°).

Sample	λ_{max} (mμ)	$\Delta\epsilon_{max} \times 10^{-3}$ (at λ)
Monoiodotyrosine	303[a]	3.6 (303)
Diiodotyrosine	311[a]	5.5 (311)
2 monoiodo- + 1 diiodotyrosine	307[a]	12.0 (310)[b] 12.0 (309)[b]
1 monoiodo- + 2 diiodotyrosine	309[a]	14.1 (310)[b] 13.8 (309)[b]
2 monoiodotyrosines	303[a]	7.2 (303)[b]
2 diiodotyrosines	311[a]	10.6 (310)[b] 10.0 (309)[b]
I_2 RNAase-C	303[c]	7.0 (303)
I_4 RNAase-B	310[d]	13.5 (310)
I_4 RNAase-C	309[d]	11.1 (309)

[a] High pH was 11.4. [b] Obtained by summing appropriate values from monoiodo- and diiodotyrosine at the wavelength in parenthesis. [c] High pH was 11.3. [d] High pH was 11.2

I_4 RNAASE-B AND I_4 RNAASE-C. Data obtained from the ultraviolet difference spectra for I_4 RNAase-B and I_4 RNAase-C are given in Figure 5 and in Table I. From the values of λ_{max}, it seems that these fractions contain both monoiodotyrosine and diiodotyrosine. To determine the compositions, we have computed the values of λ_{max} and $\Delta\epsilon_{max}$ for several combinations of monoiodotyrosine and diiodotyrosine. (Since the amount of iodine added in the iodination procedure was *less* than that required to produce I_6 RNAase, according to Woody *et al.* (1966), the calculations were carried out only for the low degrees of iodination shown in Table I.) The major component (I_4 RNAase-C) corresponds most closely to a mixture of two monoiodotyrosines and one diiodotyrosine. (We can rule out 2 diiodotyrosines on the basis (see below) that amino acid analyses and chromotograms of proteolytic digests show that I_4 RNAase-C contains *three* iodinated tyrosines.) Similarly, I_4 RNAase-B appears to contain two diiodotyrosines and one monoiodotyrosine. The fact that I_4 RNAase-B was eluted before I_4 RNAase-C from the carboxymethylcellulose probably indicates that the over-all p*K* values of fraction B are lower than those of fraction C, which is consistent with the compositions assigned above. There is no inconsistency in assigning I_4 RNAase-B an iodine content of 5 atoms per mole because the iodination need not proceed uniformly on all tyrosyl residues, especially since fraction B constituted only 25% of the total protein whereas fraction C represented 67%. The fact that λ_{max} for the iodinated derivatives is slightly higher than those for the corresponding mixtures of model compounds may be due to perturbations from neighboring groups in the protein, an effect not taken into account in the calculations.

TABLE II: Composition of Iodinated Derivatives.

Fraction	Composition
I_2 RNAase-C	2 monoiodotyrosines
I_4 RNAase-B	1 monoiodotyrosine + 2 diiodotyrosines
I_4 RNAase-C	2 monoiodotyrosines + 1 diiodotyrosine
I_6 RNAase-C	3 diiodotyrosines

The compositions of the various iodinated derivatives are summarized in Table II. Also shown in Table II is the composition of the major fraction from I_6 RNAase (*i.e.*, I_6 RNAase-C), prepared by Woody *et al.* (1966).

Amino Acid Analyses. The results of the amino acid analyses of the performic acid oxidized samples of the iodinated derivatives are shown in Table III. Since performic acid oxidation destroys both monoiodo- and diiodotyrosine (Cha and Scheraga, 1963), it can be seen from Table III that I_2 RNAase-C has four uniodinated tyrosines whereas both I_4 RNAase-B and I_4 RNAase-C have three uniodinated tyrosines. These results are consistent with the compositions given in Table II.

TABLE III: Amino Acid Analyses of Performic Acid Oxidized Iodinated Derivatives of RNAase (Moles of Amino Acid per Mole of Protein).[a]

Amino Acid	Theoretical	I_2 RNAase-C	I_4 RNAase-B	I_4 RNAase-C
Cysteic acid	8	7.52	7.82	7.48
Methionine sulfone	4	4.22	3.51	3.73
Aspartic acid	15	15.00[b]	15.00[b]	15.00[b]
Threonine	10	9.72	9.61	10.11
Serine	15	14.83	15.13	15.69
Glutamic acid	12	12.58	11.45	12.21
Proline	4	3.90	4.23	4.22
Glycine	3	3.04	2.94	3.36
Alanine	12	12.47	11.91	12.53
Valine	9	8.65	9.49	8.72
Isoleucine	3	2.15[c]	2.23[c]	1.90[c]
Leucine	2	2.10	1.85	2.07
Tyrosine	6	4.03	3.05	3.12
Phenylalanine	3	2.83	3.25	3.06
Lysine	10	9.93	10.42	10.56
Histidine	4	3.72	3.95	4.22
Arginine	4	3.93	4.00	4.54

[a] Correction factors for losses during hydrolysis: threonine 1.05, serine 1.12, cysteic acid 1.19, and tyrosine 1.14 (Rupley and Scheraga, 1963). [b] Assumed as reference. [c] Isoleucine is known to be incompletely liberated in a 22-hr hydrolysis.

Tryptic Digestion. The experiments involving tryptic and chymotryptic digestion described in this and in the next section are discussed more fully by Woody *et al.* (1966).

I_4 RNAASE-C. Proteolytic digestion was performed only on the major component (fraction C) of the I_4 RNAase derivative. The peptide elution pattern of performic acid oxidized I_4 RNAase-C was identical with that obtained for oxidized I_6 RNAase-C by Woody *et al.* (1966). The amino acid analyses of peptides O-T-2, O-T-4, and O-T-14 are presented in Table IV. Peptide O-T-2 was shifted to an earlier elution time (compared to O-T-2 from RNAase), as previously reported by Cha and Scheraga (1963), and amino acid analyses (Table IV) showed that both Tyr 73 and 76 had disappeared. Peptide O-T-4 appeared in its normal place in the elution map, and from the amino acid analyses, Tyr 25 was present, as shown in Table IV. The low yield is in accord with the results of other workers (Bailey *et al.*, 1956; Cha and Scheraga, 1963). Peptide O-T-14 also appeared in its normal position, and Tyr 92 and 97 were present in this peptide.

I_2 RNAASE-C. The elution pattern from the tryptic digest of oxidized I_2 RNAase-C was identical with that of I_4 RNAase-C (since Tyr 115 is not observable in the tryptic digest, as previously reported by Cha and Scheraga, 1963). The amino acid analyses of the tryptic peptides are presented in Table IV, from which it can be seen that the results are similar to those obtained with I_4 RNAase-C. The small amount (13% of the theoretical value) of tyrosine present in peptide O-T-2, which contains Tyr 73 and 76, may be due to a small amount of iodinated tyrosine which was not destroyed during performic acid oxidation, the iodine having been removed during acid hydrolysis (Cha and Scheraga, 1963).

Chymotryptic Digestion. I_4 RNAASE-C. The peptide elution pattern of this derivative was identical with that obtained for I_6 RNAase-C by Woody *et al.* (1966). Peptides O-C-3 and O-C-29 (both containing Tyr 115) and peptide O-C-30 (adjacent to O-C-3) all were absent from the chromatographic pattern of oxidized I_4 RNAase-C. The absence of these peptides indicates that Tyr 115 was destroyed (iodinated), and that cleavage of the bond between Tyr 115 and Val 116 did not occur.

Peptides O-C-11 (containing Tyr 25) and O-C-14 (containing Tyr 92 and 97), mixed with O-C-15, were present, whereas peptide O-C-12 (containing Tyr 73) was absent. Thus, we conclude that I_4 RNAase-C had three iodinated tyrosines (73, 76, and 115) and, in this respect, is similar to I_6 RNAase-C.

I_2 RNAASE-C. The peptide elution pattern from the chymotryptic digest of oxidized I_2 RNAase-C is shown in Figure 6. Both peptides O-C-3 and O-C-29 were present. They were isolated and analyzed (see Table V). From these data we conclude that Tyr 115 (as well as

TABLE IV: Amino Acid Analysis of O-T-2, O-T-4, and O-T-14 Obtained from a Tryptic Digest of Oxidized I_4 RNAase-C and I_2 RNAase-C, and O-T-14 Obtained from a Tryptic Digest of Oxidized RNAase (Moles of Amino Acid per Mole of Peptide).

	O-T-2 (residues 67–85)			O-T-4 (residues 11–31)			O-T-14 (residues 92–98)			
Amino Acid	Theory	I_4 RNAase-C	I_2 RNAase-C	Theory	I_4 RNAase-C	I_2 RNAase-C	Theory	RNAase	I_4 RNAase-C	I_2 RNAase-C
Cysteic acid	2	2.16	2.16	1	1.32	1.25	1	1.06	1.31	1.25
Methionine sulfone	1	1.10	0.82	3	2.65	3.13				
Aspartic acid	3	3.25	3.02	3	3.30	3.30	1	0.89	1.49	1.16
Threonine	3	3.40	2.77	1	1.40	1.30				
Serine	3	2.81	2.71	6	6.11	6.52		0.11		
Glutamic acid	2	2.01	2.08	2	2.19	2.09				0.15
Proline							1	1.11	0.75	1.06
Glycine	1	1.00[a]	1.00[a]		0.14			0.10		
Alanine			0.29	2	2.00[a]	2.00[a]	1	1.00[a]	1.00[a]	1.00[a]
Valine								0.12	0.12	
Isoleucine	1	1.01	0.71		0.18	0.22				
Leucine										
Tyrosine	2	Disappeared	0.26	1	0.59[b]	0.75[b]	2	1.90	1.80	1.81
Phenylalanine										
Lysine				1	1.16	0.95	1	0.90	0.88	0.81
Histidine				1	0.93	0.95				
Arginine	1	0.92	0.84							
Yield of peptide (%)		9	8		45	53		66	55	57

[a] Assumed as reference. [b] Low result in accord with Cha and Scheraga (1963) and Bailey *et al.* (1956).

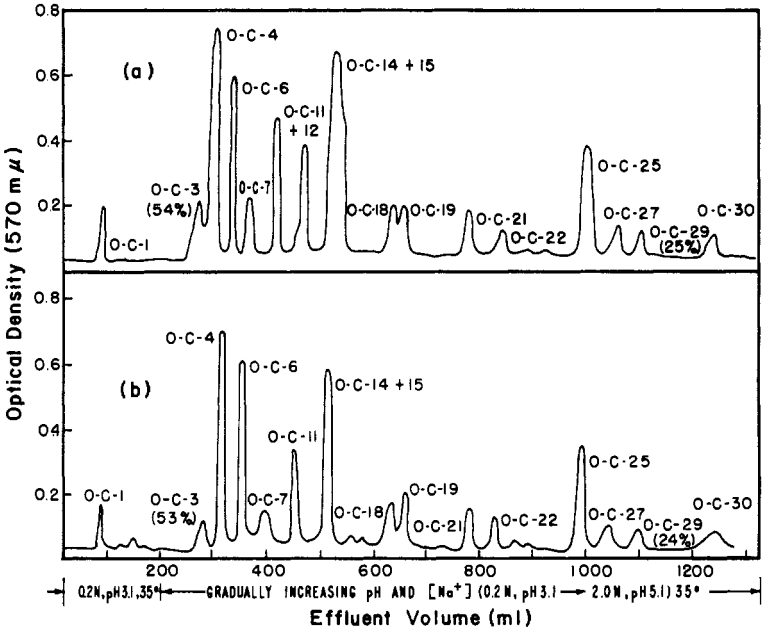

FIGURE 6: Chromatography of peptides from a 20-hr chymotryptic digest of oxidized I_2 RNAase-C on a 0.9 × 150 cm column of Dowex 50-X2. The effluent was collected in 2-ml fractions, and the ninhydrin color values were obtained with the aid of a Technicon Autoanalyzer. The percentages in parentheses represent the yields of the pertinent peptides: (a) RNAase; (b) I_2 RNAase-C.

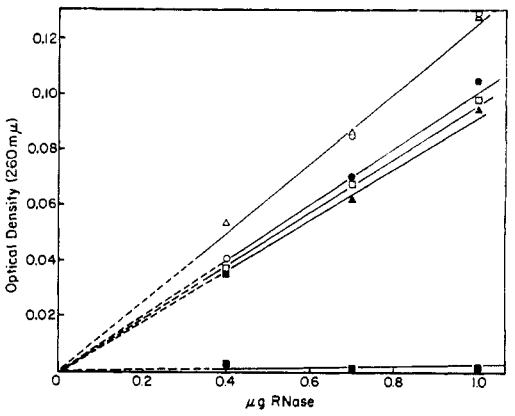

FIGURE 7: Activity measurements of RNAase, I_6 RNAase-C, and I_2 RNAase-C in 2.5 ml of solution. Initial activity of RNAase (○), of I_6 RNAase-C (□), and of I_2 RNAase-C (△). Activity of reoxidized RNAase (●), of reoxidized I_6 RNAase-C (■), and of reoxidized I_2 RNAase-C (▲).

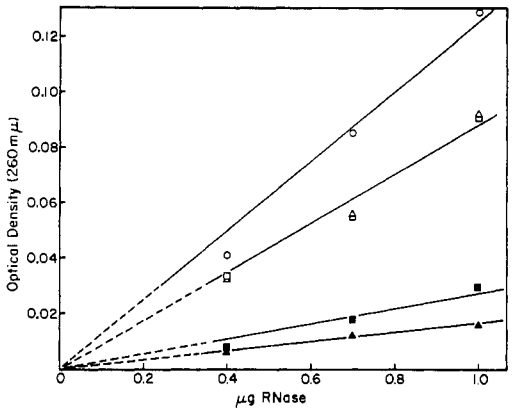

FIGURE 8: Activity measurements of RNAase, I_4 RNAase-B, and I_4 RNAase-C in 2.5 ml of solution. Initial activity of RNAase (○), of I_4 RNAase-C (□), and of I_4 RNAase (△). Activity of reoxidized I_4 RNAase-C (■) and of I_4 RNAase-B (▲). The data for RNAase (initially) are the same as shown in Figure 7.

Tyr 25, 92, and 97) was uniodinated; the only iodinated residues in I_2 RNAase-C were Tyr 73 and 76.

Activity Measurements. The results of measurements of enzymic activity, before reduction and after reoxidation, are shown in Figure 7 (for I_6 RNAase-C and I_2 RNAase-C), in Figure 8 (for I_4 RNAase-B and I_4 RNAase-C), and in Table VI. Taking the slope of the curve for RNAase (given in both Figures 7 and 8) as 100% activity, the slope for the other samples yielded the initial activities shown in the second column of

TABLE V: Amino Acid Analysis of O-C-3 and O-C-29 Obtained from a Chymotryptic Digest of Oxidized I_4 RNAase-C and I_2 RNAase-C and Oxidized RNAase (Moles of Amino Acid per Mole of Peptide).

	O-C-3 (residues 106–115)				O-C-29 (residues 104–115)			
Amino Acid	Theory	RNAase	I_4 RNAase-C	I_2 RNAase-C	Theory	RNAase	I_4 RNAase-C	I_2 RNAase-C
Cysteic acid	1	1.05		0.98	1	1.07		1.33
Methionine sulfone								
Aspartic acid	1	1.43		1.16	1	0.98		1.29
Threonine		0.36	D	0.17		0.11	D	
Serine			I	0.17		0.26	I	
Glutamic acid	1	1.03	S	1.01	1	1.00[a]	S	1.00[a]
Proline	1	0.68	A	0.88	1	1.45	A	1.00
Glycine	1	1.00[a]	P	1.00[a]	1	1.06	P	0.98
Alanine	1	0.99	P	0.95	1	1.18	P	1.11
Valine	1	0.94	E	0.96	1	0.94	E	0.88
Isoleucine	2	0.31[b]	A	0.49[b]	2	0.71[b]	A	0.50[b]
Leucine			R				R	
Tyrosine	1	0.76[c]	E	0.66[c]	1	0.61[c]	E	0.70[c]
Phenylalanine			D				D	
Lysine					1	0.95		0.88
Histidine					1	0.93		0.96
Arginine								
Yield of peptide (%)		54		53		25		24

[a] Assumed as reference. [b] Low value due to the known difficulty in hydrolyzing the isoleucine-isoleucine (106–107) bond. [c] Low value due to destruction during desalting (Hirs *et al.*, 1956b).

TABLE VI: Activity before Reduction and after Reoxidation.

Sample	Initial[a] Act. (%)	Act. of Reoxidized Sample Rel. to: Initial Act. of RNAase (%)	Act. of Reoxidized Sample Rel. to: Its Own Initial Act.[b] (%)	Column 3, × 100/80 (%)	Column 4, × 100/80 (%)
RNAase	100	80	80	100	100
I_6 RNAase-C	76	2	3	2	3
I_4 RNAase-B	78	14	18	18	23
I_4 RNAase-C	78	22	28	28	35
I_2 RNAase-C	100	74	74	93	93

[a] Before reduction. [b] Column 3 divided by column 2.

Table VI. It can be seen that I_2 RNAase-C had the same specific activity as native RNAase, and that the activities of I_6 RNAase-C, I_4 RNAase-B, and I_4 RNAase-C were slightly lower. Our earlier report (Woody *et al.*, 1966) that I_6 RNAase-C had 100% activity was based on the Kunitz (1946) assay, rather than on the method of Crestfield *et al.* (1956). The latter method, which is more sensitive, was used in this study.

Upon reduction and reoxidation, the activities shown in the third column of Table VI were obtained. In all cases, the reduced samples showed no activity. If the normalized data of the last two columns of Table VI are considered, we may conclude that full activity was regained in the cases of RNAase and I_2 RNAase-C, partial activity was regained in the cases of I_4 RNAase-B and I_4 RNAase-C, and essentially no activity in the case of I_6 RNAase-C.

Discussion

Composition of Derivatives. From the ultraviolet spectral data and from the results of amino acid analyses, it appears that the various iodinated derivatives have the composition indicated in Table II. Proteolytic digestion and chromatography of the resulting peptides led to identification of the specific tyrosyl residues which were iodinated; these are indicated in Table VII.

Whereas it was possible to identify the specific tyrosine residues that were iodinated, it was a bit more difficult to determine the degree of iodination of each tyrosyl residue. Nevertheless, it is possible to make some statements about this point. From the ultraviolet spectra, it is clear that Tyr 73 and 76 are monoiodinated in I_2 RNAase-C. This is consistent with the observed *rapid* uptake of iodine initially, especially since it is known (Mayberry *et al.*, 1965) that *N*-acetyl-L-tyrosine reacts much faster with I_2 than does monoiodinated *N*-acetyl-L-tyrosine. Also, because of this fact, it may be concluded that Tyr 115 becomes monoiodinated *before* Tyr 73 and 76 each take up their second atom of iodine. Since Tyr 73 and 76 each reacted more readily with the first atom of iodine, we suggest that these are the residues which take up the second atom of iodine; hence, I_4 RNAase-B and I_4 RNAase-C presumably contain *mono*iodinated Tyr 115, as indicated in Table VII (see also Table II). These conclusions about the state of iodination of Tyr 115 permit us to rationalize the behavior of the various derivatives in the reduction and reoxidation experiments. Also, even though we regard Tyr 73, 76, and 115 as being the three "exposed" tyrosyl residues of RNAase, it seems that Tyr 115 is probably less exposed than Tyr 73 and 76 since it is iodinated only after I_2 RNAase-C has been formed. In all of the derivatives, Tyr 25, 92, and 97 are uniodinated. These are, therefore, presumably the three tyrosines which titrate abnormally (Shugar, 1952; Tanford *et al.*, 1955).

TABLE VII: Iodinated Tyrosyl Residues in the Various Derivatives.

Derivative	Iodinated Tyrosyl Residues	State of Iodination of Residue 115
I_2 RNAase-C	73, 76	—
I_4 RNAase-B	73, 76, 115	Monoiodinated
I_4 RNAase-C	73, 76, 115	Monoiodinated
I_6 RNAase-C	73, 76, 115	Diiodinated

Reduction and Reoxidation. From the activity measurements reported in Table VI, and the compositions given in Table VII, it appears that RNAase can regain activity, after reduction and reoxidation, if Tyr 115 is *not* iodinated (as in native RNAase and I_2 RNAase-C). If Tyr 115 is diiodinated, as in I_6 RNAase-C, essentially no activity is regained. If Tyr 115 is moniodinated, as in I_4 RNAase-B and I_4 RNAase-C, partial activity is regained.

It is possible that iodination of tyrosine 115 interferes with the refolding process simply because of the steric hindrance imposed by the bulky iodine atom(s). However, we propose an alternative hypothesis. Reoxidation was carried out at pH 8.6. Data on model compounds

indicate that, at this pH, tyrosine (pK = 9.5) has its proton, diiodotyrosine (pK = 6.5) has lost its proton, and monoiodotyrosine (pK = 8.5) is half-ionized. We suggest that the protonated form of tyrosine 115 is required for the reactivation of reduced RNAase. Native RNAase and I_2 RNAase-C both have the proton on Tyr 115 and refold properly at pH 8.6. I_6 RNAase-C lacks the proton and cannot refold to the native structure. In the case of both I_4 RNAase-B and I_4 RNAase-C, one-half the molecules have the protonated form of Tyr 115; hence, a significant fraction of the activity is regenerated. It seems reasonable to assume that the pK values of the iodinated derivatives of RNAase are not significantly affected by the change in conformation of the polypeptide during refolding.

We have considered the possibility that Tyr 115 is not ordinarily involved in directing the refolding process, but can, when modified, interfere with this process (*e.g.*, the ionized form may prevent refolding). However, we feel that the most probable explanation for the data presented is that Tyr 115 contributes an essential piece of information in the process by which the polypeptide chain of reduced RNAase attains the active three-dimensional conformation. Although the nature of the specific interaction in which Tyr 115 must engage during the refolding process remains unknown, the data presented here indicate that this interaction requires that the tyrosine residue be in the protonated form.

Acknowledgment

We gratefully acknowledge the assistance of Mrs. Bonnie Dalzell in performing the amino acid analyses and of Mrs. Marilyn Meyers in performing enzyme assays.

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