Structural Studies of Ribonuclease. XXIII. Pairing of the Tyrosyl and Carboxyl Groups*

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ABSTRACT: Several observations suggest that three tyrosyl and carboxyl residues may be interacting with each other in ribonuclease. Accepting the hypothesis that such interactions may exist, and making use of the knowledge that the buried tyrosyl and carboxyl groups of ribonuclease are Tyr 25, 92, 97 and Asp 14, 38, 83, it is possible to pair these residues on the basis of studies with ribonuclease derivatives. Blocking of Asp 38 liberates Tyr 92, indicating that these residues are paired.

Liberation of the 20-residue *N*-terminus by subtilisin disrupts the Asp 14–Tyr 25 interaction. The remaining pair would then be Asp 83–Tyr 97. Ultraviolet difference spectral data on several ribonuclease derivatives provide part of the basis for the pairing of these groups.

The positions of the three buried tyrosyl groups (Woody *et al.*, 1966) and the three buried carboxyl groups (Riehm *et al.*, 1965; Riehm and Scheraga, 1966) of ribonuclease are known. However, it is not yet established whether these groups are interacting with each other and, if so, which groups are paired and what is the nature of the interactions. A series of observations are available which, by themselves, do not constitute evidence that these groups are interacting; however, taken together, it seems reasonable to postulate from these observations that the three tyrosyl groups are near three carboxyl groups, and that each of the three pairs is embedded among nonpolar groups. Part of the evidence for this postulate comes from the pH dependence of the reversible denaturation of ribonuclease (Hermans and Scheraga, 1961) and from the fact that a partially methylated derivative, having three unmodified carboxyl groups, also has three abnormal tyrosyl residues (Riehm *et al.*, 1965). In this paper, we will assume the validity of this postulate, and try to determine the way in which these groups are paired, and the structural implications of these pairings.

For this purpose, we shall consider a variety of data on several ribonuclease derivatives in which one or two of the tyrosyl-carboxyl interactions are disrupted. As with all work on protein derivatives, we assume that the tyrosyl–carboxyl interactions remaining in these derivatives also exist in the native parent protein from which the derivatives are made.

Before discussing the data on the derivatives, we shall describe some experiments with one of them, which provides the missing link, enabling us to identify all three pairs. Specifically, we have repeated the experiments of Cha and Scheraga (1963), who prepared a derivative of ribonuclease (RNAase) (in which two tyrosines were uniodinated) and identified these groups as Tyr 25 and 97. By carrying out ultraviolet difference spectral measurements on this derivative, we have been able to identify these two residues as Tyr A and C, respectively, in the nomenclature of Bigelow (1961). With this and earlier results, tyrosine residues A, B, and C become identifiable as Tyr 25, 92, and 97, respectively, enabling the proper tyrosyl and carboxyl groups to be paired.

Experimental Section

Materials. Five-times crystallized ribonuclease (lot 124B-2610) was purchased from Sigma Chemical Co. The ribonuclease A fraction was prepared by methods previously described (Rupley and Scheraga, 1963). L-Tyrosine, monoiodo-L-tyrosine, and diiodo-L-tyrosine were chromatographically pure products of Mann Research Laboratories. As_2O_3 was obtained from Mallinckrodt Chemical Works and was used to standardize the I_2 solution. All other reagents were either reagent grade or the best grade available.

Methods. PREPARATION OF IODINATED DERIVATIVES. Iodination of ribonuclease was carried out according to the procedure described by Cha and Scheraga (1963). *Ca.* 200 mg of ribonuclease A was dissolved in 10 ml of 0.5 M glycine buffer, pH 9.4. The solution was placed in the cold room (4°) and then 1.68 ml of 0.05 N I_2 solution which was 0.2 M in KI was added in 12 equivalent portions, at 5-min intervals. After the last addition of I_2, the reaction was allowed to continue for 1.5 hr before being terminated by the introduction of 1.0 ml of 4 M acetic acid. The final molar ratio of I_2:protein was *ca.* 6:1. The reaction mixture was desalted on a 3 ×

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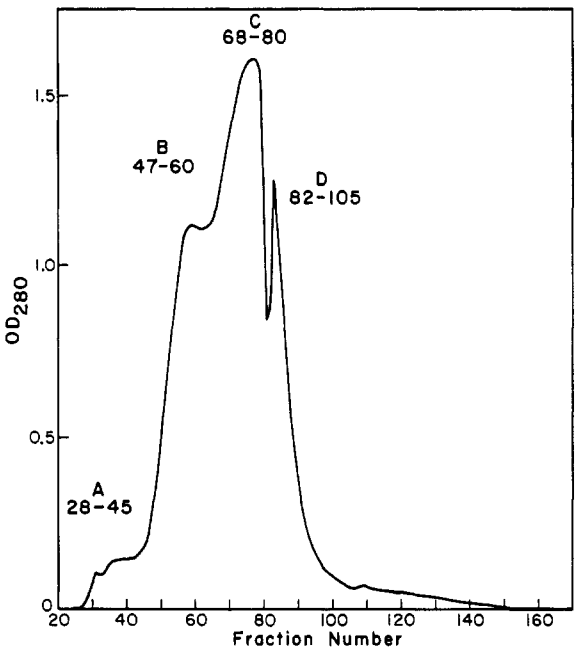

FIGURE 1: Chromatography of 110 mg of iodinated ribonuclease on a 1.5 × 40 cm Bio-Rex 70 column. Elution was made with 0.15 M phosphate buffer, pH 6.40, and collected in 2-ml fractions. Components A–D were pooled separately as shown.

40 cm column of mixed-bed ion-exchange resin (Amberlite MB-1, Mallinckrodt Chemical Works) and the column effluent was monitored with an ultraviolet flow analyzer (Canalco). The protein effluent was lyophilized to yield 110 mg and then stored in the cold room until needed.

CHROMATOGRAPHY OF IODINATED RIBONUCLEASE. Separation of the iodinated ribonucleases by column chromatography was carried out with Bio-Rex 70 resin according to the procedure described by Cha and Scheraga (1963).

PERFORMIC ACID OXIDATION. Oxidation of the iodinated ribonucleases was performed at −10°, as described by Hirs (1956).

AMINO ACID ANALYSES. Ribonuclease and the iodinated ribonucleases were hydrolyzed in 6 N glass-redistilled HCl for 22 hr in sealed and evacuated ampuls. Amino acid analyses were carried out on the automated Technicon amino acid analyzer. Corrections for hydrolysis losses were applied according to Gundlach *et al.* (1959) and Rupley and Scheraga (1963).

ULTRAVIOLET DIFFERENCE SPECTRAL MEASUREMENTS. Those measurements involving heating the protein through thermal transitions were made with a Cary Model 14 spectrophotometer, employing the general procedures described by Hermans and Scheraga (1961). Those measurements with 8 M urea present in the protein solution were made in a Zeiss spectrophotometer. The difference spectra between L-tyrosine, monoiodo-L-tyrosine, and diiodo-L-tyrosine in H_2O and these model compounds in 8 M urea were obtained by subtracting the absorption spectra in H_2O from those in 8 M urea after corrections were made for solvent absorbancies.

Results

Preparation and Separation of the Iodinated Ribonucleases. Figure 1 shows the preparative chromatogram of the iodinated proteins (from 200 mg of ribonuclease A) on a 1.5 × 40 cm column of Bio-Rex 70 resin. The equilibrating and eluting buffers were the

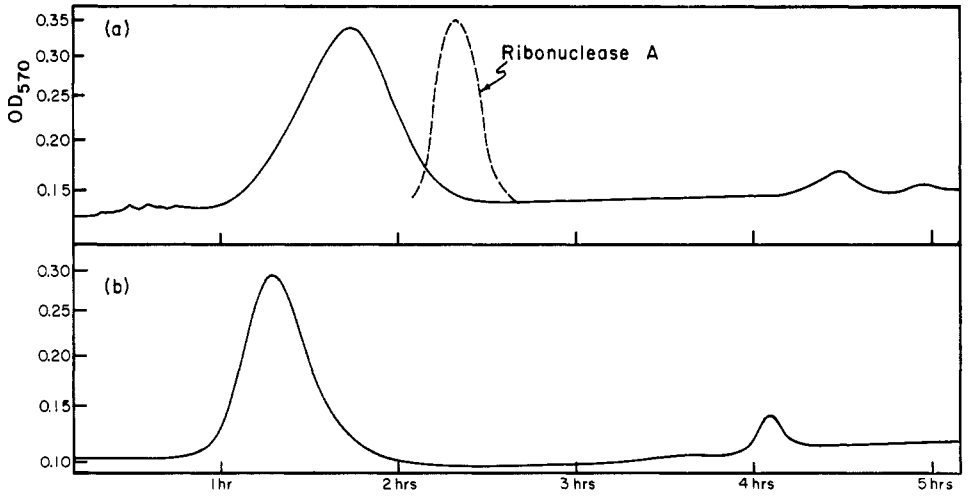

FIGURE 2: Analytical rechromatography of 2.3 mg of I-RNAase-C on a 0.9 × 30 cm column of Bio-Rex 70 (solid line) (a) and of 1.5 mg of I-RNAase-B (b). A four-stage gradient was employed as described in the text. The ninhydrin color value of the effluent (OD_{570}) was obtained at a flow rate of 20 ml/hr with the aid of a Technicon autoanalyzer. The dashed line represents a chromatogram of ribonuclease A under identical conditions.

TABLE I: Amino Acid Analysis of Iodinated Ribonuclease (Moles of Amino Acid per Mole of Protein).

Amino Acid	RNAase		I-RNAase-B		I-RNAase-C	
	Theory	Expt (Not Oxidized)	Not Oxidized	Oxidized	Not Oxidized	Oxidized
Cysteic acid	(8)			6.86		6.73
Methionine sulfone	(4)			3.67		3.72
Aspartic acid[a]	15	15.0	15.0	15.0	15.0	15.0
Threonine[c]	10	9.91	9.81	9.69	9.95	10.0
Serine[c]	15	14.9	14.1	15.0	14.8	15.0
Glutamic acid	12	12.1	12.1	12.2	12.2	12.2
Proline	4	4.34	3.34	3.52	2.85	3.15
Glycine	3	2.91	2.90	3.13	2.85	3.02
Alanine	12	12.0	12.2	12.0	11.8	11.9
Valine	9	8.94	8.20	8.37	7.95	8.28
Half-cystine[c]	8	8.19	6.11		7.35	
Methionine	4	3.12	3.38		3.24	
Isoleucine[b]	3	1.98	2.16	2.02	1.93	1.99
Leucine	2	1.88	1.84	1.93	1.93	1.87
Tyrosine[c]	6	5.63	5.32	2.56	5.65	2.42
Phenylalanine	3	2.76	2.82	2.85	2.82	2.78
Lysine	10	10.6	11.2	10.0	9.71	9.37
Histidine	4	3.70	3.44	3.67	3.20	3.07
Arginine	4	3.95	4.06	3.98	3.68	3.85

[a] Assumed reference. [b] Isoleucine is known to be incompletely released in 22 hr of acid hydrolysis. [c] Corrections for hydrolysis losses were applied for serine (1.12), threonine (1.05), tyrosine (1.14), and half-cystine (1.23) (Gundlach *et al.*, 1959), and cysteic acid (1.19) (Rupley and Scheraga, 1963).

same, *viz.*, 0.15 M phosphate, pH 6.40. Fractions (2 ml) were collected and the optical densities at 280 mμ were measured. The two major components (designated as B and C) were pooled separately, desalted on a 2.2 × 30 cm Amberlite MB-1 column, and then lyophilized. The yield of component B (I-RNAase-B) was 24 mg while that of component C (I-RNAase-C) was 41 mg. Components A and D showed considerable spreading and were recovered in amounts (5 and 20 mg, respectively), which were too small to warrant further studies.

An analytical chromatogram of I-RNAase-C, on a 0.9 × 30 cm column of Bio-Rex 70, is shown in Figure 2a. The chromatogram was developed by employing a Technicon autograd as a four-stage gradient elution device. The first three chambers contained 35 ml each of 0.15 M phosphate buffer, pH 6.40, and the fourth chamber contained 35 ml of 1.0 M phosphate buffer, pH 6.15. As may be seen from Figure 2a, the symmetrical, single peak indicates that this fraction is quite pure. Similar results were obtained with I-RNAase-B (Figure 2b).

Amino Acid Analyses. The amino acid analyses of the I-RNAase-B and I-RNAase-C hydrolysates (with or without performic acid oxidation) are shown in Table I. As would be expected, these data indicate that 6 N HCl hydrolyses of the iodinated proteins resulted in amino acid recoveries which approximated the values noted for a ribonuclease A hydrolysate. It is known that acid hydrolysis of the mono- and diiodotyrosine results in the formation of tyrosine (Cha and Scheraga, 1963). In the present studies, the yields of tyrosine residues per mole of protein for the iodinated derivatives were 5.32 and 5.65 as compared with a value of 5.63 for ribonuclease. On the other hand, since performic acid oxidation of iodinated ribonuclease results in a destruction of both mono- and diiodotyrosines (Cha and Scheraga, 1963), the number of uniodinated tyrosine residues in an iodinated derivative may be specified by determining the number of tyrosine residues recovered after acid hydrolysis of an oxidized sample. These values are 2.56 and 2.42 tyrosine residues for 1 mole of I-RNAase-B and I-RNAase-C, respectively. The yields of the remaining amino acids in these hydrolysates compared favorably, within experimental error, with those observed for the ribonuclease hydrolysate. These results indicate that both components B and C are iodinated at 3–4 tyroine residues.

Difference Spectral Measurements. Table II reports the values of difference molar extinction coefficients at 287 mμ ($\Delta\epsilon_{287}$) of several derivatives; the difference

TABLE II: $\Delta\epsilon_{287}$ of the Various Ribonuclease Derivatives Produced by Heat and by 8 M Urea.[d]

Derivative	$\Delta\epsilon_{287}$ by Heat	$\Delta\epsilon_{287}$ by 8 M Urea								
RNAase A	−1720 (A, B)	−2730 (A, B, and C)[b]								
Component H[a]	−1100 (A)	−2060 (A, C)								
		I	II	III	IV	V	VI	VII	VIII	IX
I-RNAase-B[c]	−1320 (A)	−2470	−1750	−2110	−2525	−1895	−2210	−2580	−2040	−2310
I-RNAase-C[c]	−1240 (A)	−2430	−1710	−2070	−2485	−1855	−2170	−2540	−2000	−2270

[a] Values from Table III of Riehm and Scheraga (1966). [b] Value corrected for the effect of the urea medium on normal tyrosines according to the procedure of Bigelow (1960). [c] Protein concentrations were determined by amino acid analysis (the average of the numbers of moles of the amino acids: aspartic acid, glutamic acid, glycine, alanine, and arginine, each divided by its theoretical number found in 1 l. of protein solution). Columns I–IX are the corrected values of the iodinated derivatives with the following assumptions about the composition of the six tyrosyl residues: I, 2 tyrosyl and 4 monoiodotyrosyl; II, 2 tyrosyl and 4 diiodotyrosyl; III, 2 tyrosyl, 2 monoiodotyrosyl, and 2 diiodotyrosyl; IV, 2.5 tyrosyl and 3.5 monoiodotyrosyl; V, 2.5 tyrosyl and 3.5 diiodotyrosyl; VI, 2.5 tyrosyl, 1.75 monoiodotyrosyl, and 1.75 diiodotyrosyl; VII, 3 tyrosyl and 3 monoiodotyrosyl; VIII, 3 tyrosyl and 3 diiodotyrosyl; IX, 3 tyrosyl, 1.5 monoiodotyrosyl, and 1.5 diiodotyrosyl. [d] A, B, and C in parentheses refer to the tyrosyl residues normalized by the given treatment, according to Bigelow (1961).

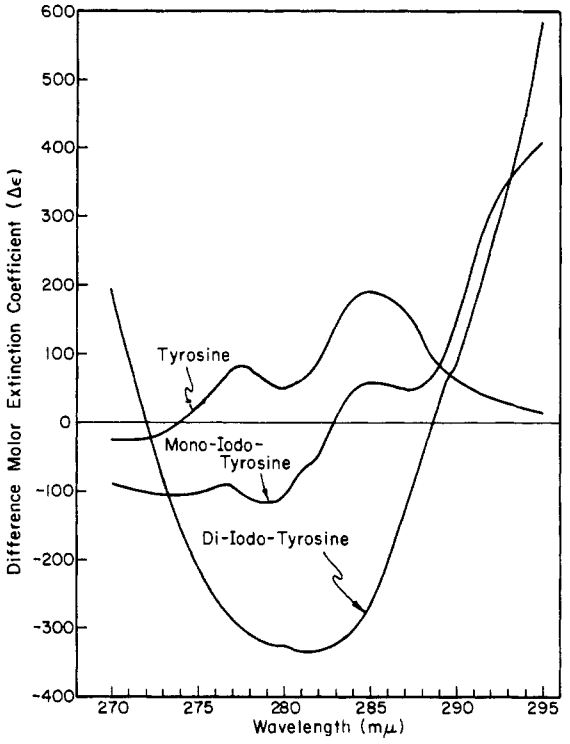

FIGURE 3: Difference spectra of L-tyrosine (4.49×10^{-4} M), monoiodo-L-tyrosine (6.10×10^{-4} M), and diiodo-L-tyrosine (2.36×10^{-4} M) produced by 8.0 M urea. The spectra were obtained by subtracting the spectrum of a given compound in H_2O *vs.* H_2O in the reference cell of the Zeiss PQ II spectrophotometer from that of the compound in 8.0 M urea *vs.* 8.0 M urea alone. The apparent pH of the solutions was between 6 and 7 and was not adjusted; however, the wavelength of maximum absorption was the same in water and in 8 M urea at these apparent pH values.

spectra were produced either by heating or by adding 8 M urea. The values for "component H"[1] are from Table III of Riehm and Scheraga (1966). The values of $\Delta\epsilon_{287}$, caused by the thermal transition of I-RNAase-B and I-RNAase-C (−1320 and −1240, respectively) agree with that of component H (−1100) within experimental error.

In 8 M urea, the observed values (not shown in Table II) were −1950 and −1910 for I-RNAase-B and I-RNAase-C, respectively. These were corrected for the effect of the urea medium on normal and iodotyrosyl residues according to the procedure of Bigelow (1960). From the difference spectra of L-tyrosine, monoiodo-L-tyrosine, and diiodo-L-tyrosine produced by 8 M urea (Figure 3) the values of $\Delta\epsilon_{287}$ (for the effect of urea on the three model compounds) are: +160, +50, and −130, respectively. The sum of the contributions from the six tyrosyl residues (per mole) of ribonuclease is, therefore, $6 \times 160 = 960$, which agrees well with the value of 860 obtained by Bigelow (1960) using data from 6 M urea. The values of the correction factors for I-RNAase-B and I-RNAase-C cannot be calculated without a knowledge of the number of di- and/or monoiodotyrosyl residues per mole of protein. However, it is possible to assume all possible combinations of the relative amounts of the various tyrosyl residues and to estimate the corresponding value of the correc-

[1] This derivative was prepared (Riehm and Scheraga, 1966) by reacting ribonuclease with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate. In one of the reaction products (component H) one of the three abnormal tyrosyl residues was normalized after reaction of Asp 38 with the carbodiimide. Using the ultraviolet difference spectral technique of Bigelow (1961), Riehm and Scheraga identified the normalized tyrosyl residue as Tyr B. The remaining two tyrosyl residues (Tyr A and C) of component H remained abnormal. Thus, Asp 38 is probably paired with Tyr B.

TABLE III: Tyrosyl Groups in Ribonuclease Which are Normalized upon Denaturation.[e]

	by Heat			by Urea		
	$\Delta\epsilon_{287}$	Groups Normalized	Groups Still Buried	$\Delta\epsilon_{287}$	Groups Normalized	Groups Still Buried
RNAase A	−1720	A (25), B (92)	C (97)	−2730	A (25), B (92), C (97)	None
RNAase-S[a]	−1700	A (25), B (92)	C (97)	−2700	A (25), B (92), C (97)	None
PIR[b]	−1000	A (25)	None	−1000	A (25)	None
Derivative H[c]	−1100	A (25)	C (97)	−2060	A (25), C (97)	None
Iodinated RNAase	−1240	A (25)	C (97)	(−2000)[d]	A (25), C (97)	None
S-Protein[a]	−800	B (92)	C (97)	−1710	B (92), C (97)	None

[a] Data from Sherwood and Potts (1965). [b] Data from Bigelow (1960). [c] Data from Riehm and Scheraga (1966). [d] See Discussion section for assignment of this value. [e] In all cases, the denatured protein is measured against the native protein. The data for urea denaturation are corrected for the effect of the urea medium.

tion for each combination using the observed values of $\Delta\epsilon_{287}$ of the three model compounds. By subtracting these values from the observed $\Delta\epsilon_{287}$ of the I-RNAase-B (−1,950) and the I-RNAase-C (−1910) the corrected values in columns I–IX of Table III were obtained.

Discussion

Analytical Data. Analytical chromatography and amino acid analysis data of the iodinated derivatives which were isolated in the present experiments indicate that they are essentially the same as those studied by Cha and Scheraga (1963). The slightly higher values for the tyrosine content (2.56 and 2.42 as compared to 2.34 and 2.43 reported by Cha and Scheraga) recovered from 6 N HCl hydrolyses of the performic acid oxidized derivatives suggests a slightly lower iodine content in our derivatives. This may be due to a difference in the procedure used for desalting the reaction mixture after iodination. Exhaustive dialysis was used by Cha and Scheraga, whereas an Amberlite MB-1 column was employed in the present investigation. The relatively low yields (24 mg of I-RNAase-B and 41 mg of I-RNAase-C from 200 mg of ribonuclease A) are presumably also a result of desalting on the mixed-bed ion-exchange resin. Having shown the identity of our derivatives and those of Cha and Scheraga, we rely on their peptide analyses to identify the *two* buried tyrosyl groups as Tyr 25 and 97.

Spectral Data. Bigelow (1961) showed that heat normalizes only two tyrosines (designated Tyr A and B), whereas the third tyrosine (Tyr C) becomes normalized only in 8 M urea (or under conditions, such as oxidation of the disulfide bridges, which are as disruptive of the native structure). He assigned values of −1000, −700, and — 1000 to Tyr A, B, and C, respectively, as the change in molar extinction at 287 mμ when the environment of the protein is changed.

Since the values of $\Delta\epsilon_{287}$ for I-RNAase-B, I-RNAase-C and component H are −1320, −1240, and −1100, respectively, for the thermal transition, it appears that only one tyrosine (*i.e.*, Tyr A) is normalized in this treatment.[2] It thus appears that Tyr B was normal in these three derivatives, even prior to the heating.

Since the addition of urea can cause the normalization of Tyr A, B, and C, the *increments* in 8 M urea must be attributed to the normalization of Tyr C. This accounts for the values of −2060 for component H and for the values near −2000 (independent of the assumption made about the composition of the tyrosines) for I-RNAase-B and I-RNAase-C. Marked deviations from a value of −2000 are found only in cases where an appreciable amount of the modified tyrosyl residues are assumed to be in the monoiodo form. It is highly unlikely that the modified tyrosyl residues of I-RNAase-B and I-RNAase-C are in the monoiodo form, since the iodination of *N*-acetyl-3-iodo-L-tyrosine at pH 9.80, 20°, was found to be 75% completed within 25 min (Mayberry *et al.*, 1965).

We thus conclude that the two buried tyrosines in both the two iodinated derivatives studied here and also in component H are Tyr A and C. Since Cha and Scheraga (1963) identified these as Tyr 25 and 97, we conclude that Tyr B, the one that is near Asp-38 (Riehm and Scheraga, 1966) is the third buried tyrosine of ribonuclease, iodinated in the experiments of Cha and Scheraga (1963) but not in those of Woody *et al.* (1966); according to Woody *et al.* (1966) Tyr B is Tyr 92.

Structural Implications. From the above, it seems that Tyr 92 (Tyr B) could be paired with Asp 38 since

[2] The slightly higher values observed during the thermal transitions of I-ribonuclease-B and I-ribonuclease-C, as compared with that of component H, may be due to a small contribution from the iodotyrosines. This is likely the explanation since the difference spectrum of I-ribonuclease-C at pH 6.7 during the heat transition (Figure 4a) shows a broad shoulder above 290 mμ which is not observed in ribonuclease A (Figure 4b). A similar phenomenon has been observed with I-ribonuclease-B (L.-K. Li, J. P. Riehm, and H. A. Scheraga, unpublished results).

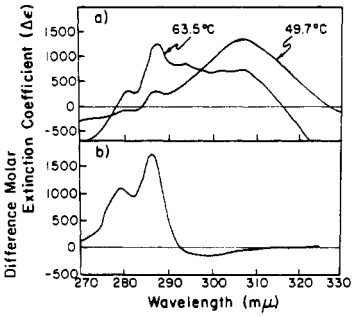

FIGURE 4: Ultraviolet difference spectra during thermal transition of: (a) I-RNAase-C in 0.15 M KCl at pH 6.7. The temperature of the sample cell was 17° in the Cary Model 14 recording spectrophotometer while that of the reference cell varied as shown. The protein concentration (for computing $\Delta\epsilon$) was determined by amino acid analysis of a given volume of the protein solution. (b) Ribonuclease A at pH 6.9. The temperature of the reference cell was 67.9° and that of the sample cell was 17°.

tyrosyl residue B is normalized upon modification of Asp 38 in component H (Riehm and Scheraga, 1966). Similar considerations can be used to obtain the remaining pairings, on the assumption that the Tyr and Asp groups do indeed interact with each other.

Pepsin-inactivated ribonuclease (PIR)[3] has one buried tyrosine, which is Tyr 25 (Fujioka and Scheraga, 1965). Bigelow (1960) showed that the one buried tyrosine in PIR is Tyr A (see Table III). Hence, Tyr A is Tyr 25 and the remaining buried tyrosine (Tyr 97), identified by Cha and Scheraga (1963), must be Tyr C. The spectral behavior, summarized in Table III, is consistent with these identifications.

From the recent work of Sherwood and Potts (1965), RNAase-S has a spectral behavior similar to that of RNAase A (see Table III). However, S-protein (lacking the 20-residue N-terminus) has only two buried tyrosines (Tyr B and C, according to Sherwood and Potts; see also Table III). In other words, Tyr A (*i.e.*, Tyr 25) is normalized by removal of the N terminus. Since the only buried carboxyl group in the N terminus is Asp 14 (Riehm *et al.*, 1965), we conclude that Tyr 25 is paired with Asp 14. Since the remaining buried carboxyl group of ribonuclease is Asp 83 (Riehm *et al.*, 1965), it is probably paired with Tyr 97.

In summary, we propose the following pairings in native ribonuclease: Tyr 25–Asp 14, Tyr 92–Asp 38, and Tyr 97–Asp 83. The C terminus must lie in the neighborhood of the Tyr 92–Asp 38 and Tyr 97–Asp 83 pairs, since liberation of the C-terminal tetrapeptide by pepsin normalizes Tyr 92 and 97 (Fujioka and Scheraga, 1965). This constraint will also bring His 119 near Lys 41. The well-known interaction of the N- and C termini would bring His 12 into proximity with His 119 and Lys 41 to form the active site. Based on these and other considerations, a model of ribonuclease has been constructed (in collaboration with G. Hammes). Work is now in progress to locate the interacting carboxyl group(s) in PIR to provide independent evidence on the above pairing of the buried tyrosyl and carboxyl groups.

[3] Abbreviations used: PIR, pepsin-inactivated ribonuclease.

Acknowledgment

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