Use of a Nonenzymatic Cleavage Reaction for the Identification of Exposed Tyrosine Residues in Bovine Pancreatic Ribonuclease†

Yigal Burstein and Abraham Patchornik*

ABSTRACT: A method of possible general utility for the location of buried and exposed tyrosine residues in proteins has been developed using bovine pancreatic ribonuclease as a model. Exposed tyrosine residues were acetylated with *N*-acetylimidazole or acetic anhydride and were thus protected from cleavage by *N*-bromosuccinimide. The partially acetylated protein was reduced with dithioerythritol, S-carboxymethylated with 2-iodoacetic acid, and subjected to cleavage with *N*-bromosuccinimide. By determining the bonds that could be cleaved with *N*-bromosuccinimide, tyrosine residues that were buried in the native molecule could be identified. The method was applied to partially acetylated ribonuclease. *N*-Bromosuccinimide cleavage and end-group analysis of the cleaved peptide bonds revealed that tyrosine residues 25, 92, and 97 were the only ones to be cleaved, and it was thus concluded that the other three tyrosine residues, namely, residues 73, 76, and 115, had been acetylated and therefore not cleaved by *N*-bromosuccinimide.

An important step in the study of a protein molecule in solution is to determine which amino acid side chains are exposed to the solvent and which are buried in the interior of the molecule. Since Cramer and Neuberger (1943) have introduced spectrophotometric titrations of tyrosine residues in proteins, this method has been extensively used for the determination of "exposed" and "buried" tyrosine residues in many proteins including RNase.[1] Shugar (1952) and Tanford *et al.* (1955) found that in native RNase three of the six tyrosine residues ionize normally (and reversibly) in the range of pH 9–11.5, and the other three ionize abnormally (and irreversibly) above pH 11.5. These anomalous tyrosines are not equivalent as indicated by differences in the spectral shifts occurring under various denaturation conditions (Bigelow, 1961) and by solvent perturbation measurements (Hershkovitz and Laskowski, 1968). Chemical modifications of tyrosine residues in RNase support these findings. Cyanurylation (Gorbunoff, 1967; Takenaka *et al.*, 1967), nitration (Sokolovski *et al.*, 1966), and acetylation (Simpson and Vallee, 1966; Riordan and Vallee, 1967), affected only three of the six tyrosine residues of the native enzyme. When RNase was iodinated under mild conditions (Cha and Scheraga, 1963a,b; Donovan, 1963; Woody *et al.*, 1966), again only three tyrosine residues were iodinated and identified as residues 73, 76, and 115 in native RNase.

In this paper we describe a simple method for the location of acetylated tyrosine residues in proteins. This method is based on the findings that tyrosine peptide bonds are known to be cleaved by NBS under acidic conditions (Schmir *et al.*, 1959; Schmir and Cohen, 1961; Wilson and Cohen, 1963a,b) and the cleavage is prevented by O-acetylation of the phenol moiety (Shaltiel and Patchornik, 1961, 1963). We applied this method to bovine pancreatic RNase, and identified the three residues which are normally acetylated in the native enzyme.

Materials

Bovine pancreatic ribonuclease A (five-times recrystallized, type 1A lot no. 95B-0330) and Tris, reagent grade, were purchased from Sigma Chemical Co. Proline iminopeptidase was a gift of Mr. D. Mlynar of our department. Ribonucleic acid (yeast nucleic acid lot no. 6502) was obtained from Schwarz BioResearch, Inc. Dithioerythritol (lot no. 5531) was a product of Cyclo Chemical Corp. Analytical reagent grade urea, obtained from BDH Ltd. was recrystallized from 95% ethanol; freshly prepared solutions were used. NBS was a product of Fluka AG, and was recrystallized from water. *N*-Acetylimidazole was purchased from K & K Fine Chemicals. DNP-amino acids were a product of Mann Research Labs., Inc. Performic acid oxidized ribonuclease was prepared according to Hirs (1967).

Methods

Spectrophotometric measurements were made with a Cary Model 14 spectrophotometer. Quartz cells with 1-cm light path were used.

RNase concentrations were determined spectrophotometrically at 277.5 nm using $E_{277.5} = 9800$ l. mole^{-1} cm^{-1} (Sela and Anfinsen, 1957).

pH-Stat titrations were carried out in an all-glass thermostated reaction vessel, using a Radiometer type TTT1c automatic titrator in combination with TTA3 titration assembly and SBR_2-SBU1 titrigraph recorder. The electrodes were the G-2222B glass electrode and the K4112 calomel electrode (Radiometer, Copenhagen).

Amino acid analyses were performed on a Beckman-Spinco automatic amino acid analyzer Model 120C, after hydrolysis in 6 N HCl for 22 hr at 110° (Spackman *et al.*, 1958).

Acrylamide disc electrophoresis was performed on a Shandon apparatus, using 15% gels, pH 4.3 for 90 min according to Reisfeld *et al.* (1962).

RNase activity on RNA was measured spectrophotomet-

† From the Department of Chemistry, The Weizmann Institute of Science, Rehovot Israel. *Received April 24, 1972.* This research was supported in part by U. S. Public Health Service Grant No. AM05098.

[1] Abbreviations used are: NBS, *N*-bromosuccinimide; RNase, bovine pancreatic ribonuclease; Ribox, performic acid oxidized RNase; 8CM-RNase, RNase derivative in which all four disulfide bonds were reduced and carboxymethylated; Ac_3-RNase, RNase derivative in which three tyrosine residues were acetylated; FDNB, 2,4-dinitrofluorobenzene; CM-cysteine, *S*-carboxymethylcysteine.

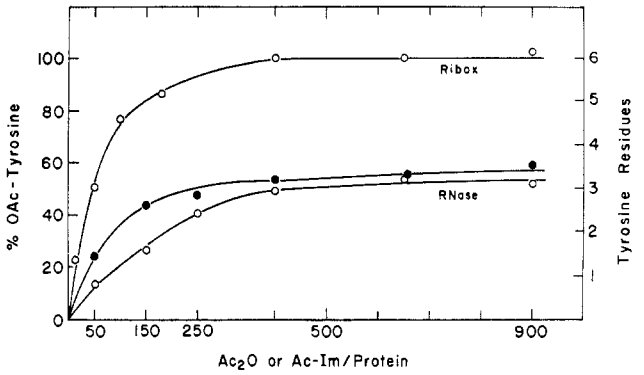

FIGURE 1: Acetylation of tyrosine residues in ribonuclease (RNase) and in performic acid oxidized RNase (Ribox) with (○) *N*-acetylimidazole (Ac-Im), and (●) acetic anhydride (Ac_2O). The reactions were carried out at 0° for 45 min. For details, see text.

rically according to the procedure of Kunitz (1946), at 25° in 0.1 M acetate buffer (pH 5.0).

Preparation of Acetylated RNase. ACETIC ANHYDRIDE METHOD. RNase (0.5–1.5 μmoles/ml) was dissolved in 2–4 ml of water in a reaction vessel of the pH-Stat. The reaction vessel was kept at 0° with constant stirring. The solution was brought to pH 7.5 and acetic anhydride in dioxane (1:1) was added. The pH was kept constant by the use of 1 M NaOH. The reaction was carried out for 45 min and was terminated by the addition of a few drops of concentrated HCl. The reaction mixture was immediately applied onto a Sephadex G-25 column (2 × 80 cm) and eluted with 0.2 M acetic acid at a rate of 45 ml/hr. The protein fraction was pooled and lyophilized.

N-ACETYLIMIDAZOLE METHOD. RNase (0.5–1.5 μmoles/ml) was dissolved in 2–4 ml of Veronal buffer (0.01–0.02 M, pH 7.5) with constant stirring at 0°. Acetylimidazole in minimum dioxane was added and the reaction was carried out for 45 min. The modified protein was purified as described above.

Determination of O-Acetyltyrosine Residues in RNase. ALKALINE HYDROLYSIS. Determinations were carried out according to Shaltiel and Patchornik (1963). Equal volumes of the reaction mixtures in 8 M urea, were placed in two cells of the spectrophotometer. The solution in the sample cell was brought to pH 12 with concentrated NaOH and an equal volume of water was added to the reference cell. The optical density at 295 nm was measured after 5–10 min, and the total tyrosine content was determined using $E_{295} = 2630$ l. mole^{-1} cm^{-1} (Tanford *et al.*, 1955). The solution in the sample cell was then brought back to pH 7 with concentrated HCl, and again an equal volume of water was added to the reference cell. The optical density at 280 nm was measured and the concentration of the deacetylated tyrosine residues was determined using $\Delta E_{278} = 1280$ l. mole^{-1} cm^{-1} (Shaltiel and Patchornik, 1963).

HYDROLYSIS WITH HYDROXYLAMINE. Determinations were carried out according to Simpson *et al.* (1963). To the protein solution at pH 7.5, a freshly prepared solution of hydroxylamine (2–3 M, pH 7.5) was added to yield a final concentration of 0.1–0.5 M. The change in the absorption at 278 nm was recorded for 10–30 min (until no change in the absorption could be observed), and the concentration of the *O*-acetyltyrosine residues was determined using $E_{278} = 1160$ l. mole^{-1} cm^{-1}.

NBS Cleavage of RNase Derivatives. The protein (0.2–0.5 μmole/ml) was dissolved in pyridine–acetic acid buffer, pH 3.3 (pyridine–acetic acid–water, 1:10:19), and to this solution NBS in the same buffer was added. After 30–60 min at room temperature, the reaction was terminated by the addition of excess sodium thiosulfate, the solvent was evaporated and the residue was redissolved in 2.0 ml of water.

Determination of the Yields of Cleavage of Tyrosyl Peptide Bonds in RNase Derivatives. DNP METHOD. One milliliter of the above cleavage mixture was adjusted to pH 8 with sodium bicarbonate, and treated with fluorodinitrobenzene according to the procedure summarized by Frankel-Conrat *et al.* (1955). The mixture of DNP-amino acids thus formed was separated into two fractions. Ether-soluble DNP-amino acids were separated by two-dimensional thin-layer chromatography on 20 × 20 cm silica gel plates developed with toluene–pyridine–2-chloroethanol–0.8 M ammonia (10:3:6:6, upper phase) in the first dimension and chloroform–methanol–acetic acid (95:5:1) in the second dimension. Acid-soluble DNP-amino acids were separated by high-voltage paper electrophoresis in 10% acetic acid 60 V/cm for 3 hr (on a Shandon cool-plate apparatus). The amount of each DNP-amino acid was determined by elution of the corresponding yellow spots and spectrophotometric assay. The results were corrected for hydrolytic and chromatographic losses.

PROLINE IMINOPEPTIDASE METHOD. One-half a milliliter of the cleavage reaction mixture (0.5–1 μmole) was adjusted to pH 8 with 0.1 M Veronal buffer, and 50 μl of 0.05 M $MnCl_2$ and 100–200 μg of proline iminopeptidase were added. The reaction mixture was incubated at 37° for 24 hr and the reaction was stopped by boiling for 2 min. To 1.0 ml of the incubation mixture 2.5 ml of glacial acetic acid and 2.5 ml of acidic ninhydrin solution (75 mg of ninhydrin in 1.5 ml of glacial acetic acid and 1.0 ml of 70% orthophosphoric acid) were added. The mixture was boiled for 1 hr and proline was determined spectrophotometrically at 530 nm (Sarid *et al.*, 1959, 1962).

Determination of N-Acetyllysine Residues in Acetylated RNase Derivatives. DEAMINATION METHOD (according to Anfinsen *et al.*, 1962). Acetylated protein (2–5 mg) was dissolved in 0.5 ml of water, and to it 1.5 ml of a saturated solution of $NaNO_2$ and 0.5 ml of glacial acetic acid were added. The reaction was allowed to proceed overnight at room temperature, and the protein was then desalted on a Sephadex G-25 column. The protein peak was pooled, lyophilized, and *N*-acetyllysine residues were determined by automatic amino acid analysis after acid hydrolysis. In a control experiment, 9.7 lysine residues (out of 10) in native RNase were destroyed under these reaction conditions; diacetyllysine was not affected at all.

Results

Acetylation of RNase. RNase was acetylated with acetic anhydride or with *N*-acetylimidazole at pH 7.5. The extent of acetylation of tyrosine residues in acetylated RNase was determined spectrophotometrically according to Shaltiel and Patchornik (1963) and Simpson *et al.* (1963). The results are illustrated in Figure 1. As was previously shown by Shaltiel and Patchornik (1963) and by Simpson *et al.* (1963), only about 50–60% of the tyrosine residues in native RNase could be acetylated with acetic anhydride or with *N*-acetylimidazole, and quantitative acetylation of these residues could be achieved with performic acid oxidized RNase. The extent of acetylation of lysine residues in native RNase was also determined. Two different methods were employed. (a) The nonacetylated lysine residues in partially acetylated RNase were deaminated according to Anfinsen *et al.* (1962) and the acet-

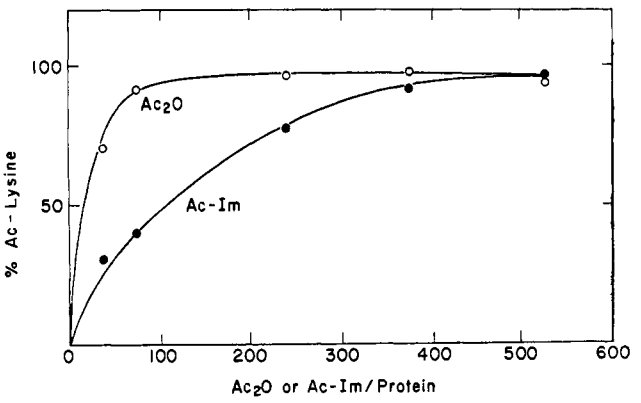

FIGURE 2: Acetylation of lysine residues in RNase with (○) acetic anhydride (Ac_2O) and (●) *N*-acetylimidazole (Ac-Im). For details, see text.

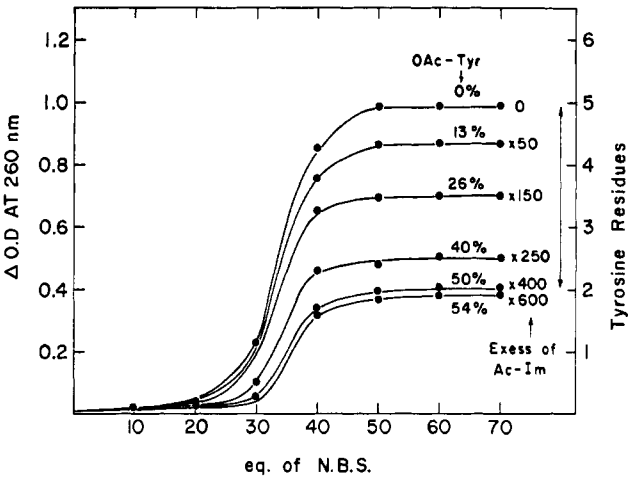

FIGURE 3: NBS titrations of partially acetylated RNase derivatves. To 3.0 ml of acetylated RNase derivative (2×10^{-5} M) in 0.1 M H_2SO_4 in the spectrophotometer cell, 10 μl of NBS (6×10^{-2} M) in dioxane was added first to the reference cell and then to the sample cell. The change in the optical density at 260 nm was recorded.

ylated lysine residues were determined by automatic amino acid analysis after hydrolysis. (b) The acetylated protein was dinitrophenylated and hydrolyzed, and N^{α}-DNP-lysine, N^{ϵ}-DNP-lysine, and N^{α},N^{ϵ}-bis-DNP-lysine were isolated and determined as described under Methods. In this method the *nonacetylated* lysine residues were determined. The results are shown in Figure 2. As can be seen from this figure, almost quantitative acetylation of lysine residues in RNase can be obtained with either acetic anhydride or *N*-acetylimidazole. While acetic anhydride can block 95% of the lysine residues in native RNase when used in a relatively small excess (100 moles/mole of protein), *N*-acetylimidazole under the same reaction conditions, can block only about 50% of the lysine residues in the native enzyme. We chose therefore to use *N*-acetylimidazole in the acetylation reaction.

Determination of O-Acetyltyrosine Residues with the NBS Method. As was shown by Corey and Haefele (1959) and Schmir *et al.* (1959) tyrosine residues are converted with NBS to dibromodienone-spirolactone residues, leading to the cleavage of the tyrosyl peptide bond. The formation of this intermediate structure causes a decrease in the absorption at 280 nm and an increase in the absorption at 260 nm, E_{260} 10,200 l. mole^{-1} cm^{-1} (Schmir *et al.*, 1959; Schmir and Cohen, 1961). *O*-Acetyltyrosine residues cannot undergo this lactonization[2] and therefore do not contribute to the increase in the absorption at 260 nm (Shaltiel and Patchornik, 1963). We used these findings for the determination of the remaining free tyrosine residues in partially acetylated RNase derivatives. Acetyl-RNase derivatives (with different degrees of acetylation) were reacted with NBS and the increase in the absorption at 260 nm was recorded as a function of NBS concentration. The results are illustrated in Figure 3. As can be seen from this figure, about 50–60 equiv of NBS was needed in order to obtain maximum increase in the absorption at 260 nm indicating that other oxidation-sensitive amino acids consumed some NBS. In the native enzyme (0% *O*-Ac-Tyr) only five out of the six tyrosine residues were converted to the dienone lactone derivatives, and exhibited the expected increase in the absorption at 260 nm. This phenomenon was explained by Wilson and Cohen (1963b), who found out that in this particular case, tyrosine-25 in RNase is not converted to the dibromodienone lactone derivative and therefore was not cleaved by NBS. However, the most striking finding is the fact that the higher the degree of acetylation of tyrosine residues in RNase, the smaller the increase in the absorption at 260 nm. The difference in the *increase* in this value when the native enzyme (upper curve) is compared to the enzyme in which 54% of its tyrosine residues was acetylated (lower curve) is equal to three acetylated tyrosine residues.

Homogeneity Studies of Acetyl-RNase. Acetyl-RNase (45% *O*-acetyltyrosine residues) was subjected to ion-exchange chromatography on an SE-Sephadex column at pH 6.47. The elution pattern is illustrated in Figure 4. As can be seen from this figure, several peaks were obtained; peak II was eluted at the position of the native enzyme while peak I (over 90% of the protein) was eluted ahead of it. Peak I contained very little enzymatic activity, and was inhomogeneous as was judged by its general shape (absorption at 280 nm), but surprisingly enough its *O*-acetyltyrosine content was fairly homogeneous (44–52%) along the whole peak. We as-

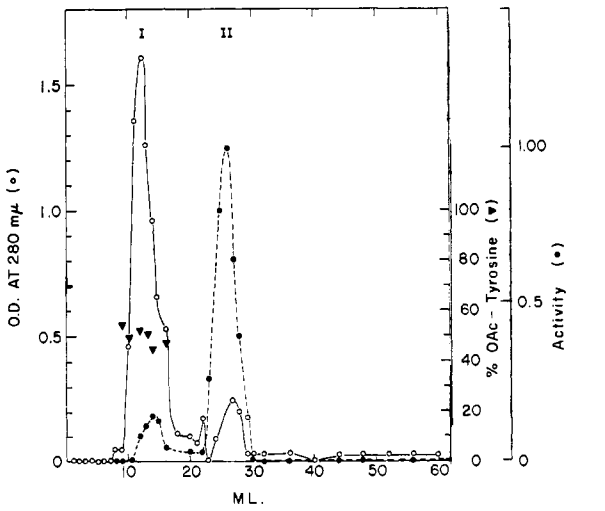

FIGURE 4: Ion-exchange chromatography of AC_3-RNase. AC_3-RNase (1.5 μmoles) was separated on a SE-Sephadex C-25 column (1.0 × 45 cm) developed with 0.2 M phosphate buffer (pH 6.47) at a rate of 15 ml/hr, 2 ml/fraction; (○) absorption at 280 nm, (●) activity (Kunitz, 1946), (▼) per cent of *O*-acetyltyrosine residues.

[2] Although modification of *O*-acetyltyrosine does occur.

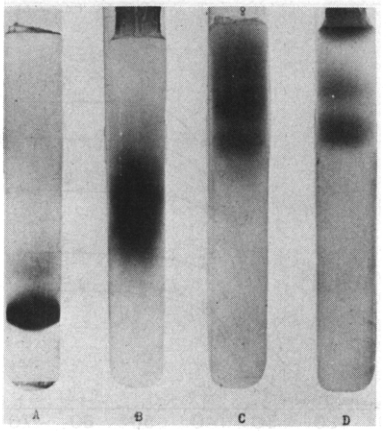

FIGURE 5: Gel electrophoresis of partially acetylated RNase derivatives. Electrophoresis was carried out in 15% gels, at pH 4.3, for 90 min, 6 mA/tube according to Reisfeld *et al.* (1962). A native RNase; B, C, and D, RNase derivatives acetylated with 150, 300, 600 equiv of *N*-acetylimidazole, respectively.

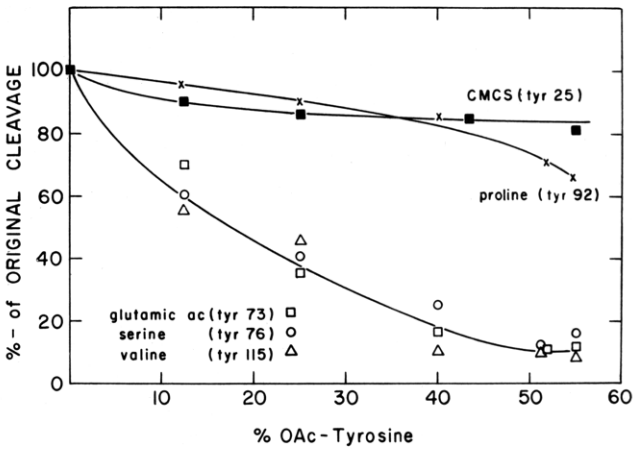

FIGURE 7: Cleavage yields of tyrosyl peptide bonds in RNase derivatives. RNase derivatives (0.5–1.5 μmoles/ml) were cleaved with 60 equiv of NBS in pyridine–acetic acid buffer (pH 3.3) at room temperature for 60 min. Yields were determined by end-group analysis of the new N-terminal amino acids. For details, see text.

sumed that the inhomogeneity could be mainly due to different degrees of acetylation of lysine residues. This assumption was tested by subjecting several acetyl-RNase derivatives to disc electrophoresis at pH 4.3. As shown in Figure 5, the higher the degree of acetylation the greater the retardation of derivatives on the gels. These studies indicate that an RNase derivative in which about 50% of the tyrosine residues were acetylated with *N*-acetylimidazole (Ac_3-RNase), is not homogeneous with respect to its *N*-acetyllysine content, but is fairly homogeneous with respect to its *O*-acetyltyrosine content.

Reduction of the Disulfide Bonds in Acetylated RNase. Wilson and Cohen (1963b), have shown that NBS does not cleave the tyrosyl–cysteine bond (residues 25–26) in RNase. This bond could be cleaved with NBS in the reduced and alkylated enzyme. Reduction of the disulfide bonds in acetyl-RNase with mercaptoethanol in urea or with sodium borohydride hydrolyzed the *O*-acetyl residue, but dithioerythritol in urea could be used for this purpose. Figure 6 illustrates the course of the reduction of RNase in urea as a function of the concentration of dithioerythritol at pH 8. As can be seen from this figure the reduction was quite efficient at a dithioerythritol concentration of 10 mM, and about 90% of the disulfide bonds was reduced within 30 min. By increasing the concentration of dithioerythritol to 20 mM, quantitative reduction of the disulfide bonds in RNase derivatives could be obtained within 15 min with less than 5% hydrolysis of the *O*-acetyltyrosine moiety.

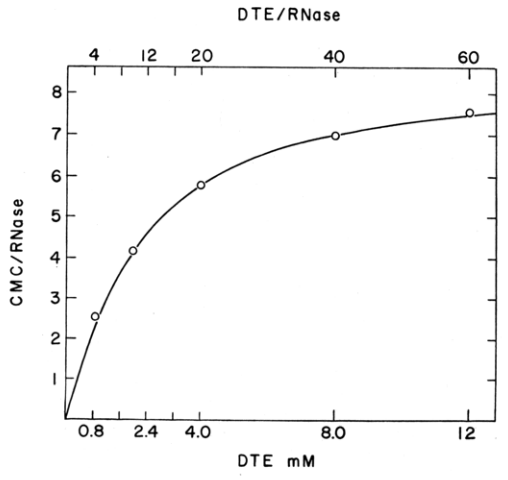

FIGURE 6: Reduction of RNase with dithioerythritol (DTE). RNase (0.2 μmole/ml) in 0.1 M Tris-HCl buffer (pH 8.0) and 8 M urea, was reduced with increasing concentrations of DTE at room temperature. After 30 min iodoacetic acid (5 equiv) was added for 15 min and the modified protein was purified by gel filtration. CM-cysteine residues were determined by automatic amino acid analysis.

Nonenzymatic Cleavage of Tyrosyl Peptide Bonds in Acetylated RNase. Several acetylated RNase derivatives were reduced with dithioerythritol and the free sulfhydryl groups were carboxymethylated with iodoacetic acid. The acetylated reduced and carboxymethylated RNase derivatives were then cleaved with NBS at pH 3.3 for 60 min. The new amino terminals thus formed were determined quantitatively by the DNP method (*S*-carboxymethylcysteine sulfone, glutamic acid, serine, valine, and lysine), and N-terminal proline was determined by the proline iminopeptidase method. The results are summarized in Figure 7. From these results it appears that the higher the degree of acetylation of the tyrosine residues in RNase, the lower were the yields of cleavage next to tyrosine residues 73, 76, and 115. The yield of cleavage next to tyrosine 25 remained constant (up to 54% *O*-acetyltyrosine residues) and the yield of cleavage of the peptide bond next to tyrosine-92 started to drop only after about 35% of the tyrosine residues were acetylated. The cleavage next to tyrosine-97 exposed the α-amine of lysine-98. Since some of the lysine residues in RNase were also acetylated by *N*-acetylimidazole, two new N-terminal amino acids could be expected at this position, an N-terminal lysine or an N-terminal N^{ϵ}-acetyllysine. The same considerations should be applied to the fact that the N-terminal residue of this protein is also lysine, and therefore three N-terminal amino acids in the partially acetylated enzyme should be considered: lysine, N-$^{\alpha}$-acetyllysine and N^{ϵ}-acetyllysine.[3] Therefore, in order to determine the yield of cleavage next to tyrosine-97, the α-amine of lysine[4]

[3] A fourth derivative, N^{α},N^{ϵ}-diacetyllysine could be formed, but such a residue would not yield a DNP derivative.

[4] α-Aminolysine was determined by the DNP method; it was the sum of N^{α}DNP-lysine and N^{α},N-$^{\epsilon}$bis-DNP-lysine.

must be determined *before* and *after* cleavage with NBS. The difference between these values was used for the determination of the yields of cleavage of this peptide bond.

Table I summarizes the yields of cleavage of the tyrosyl peptide bonds in nonacetylated RNase and in an RNase derivatives in which 54% of the tyrosine residues was acetylated by *N*-acetylimidazole. As can be seen from this table, the cleavage next to tyrosine residues 25 and 97 was hardly affected by the acetylation of the enzyme. The cleavage next to tyrosine-92 was reduced to about two-thirds of its original yield in the native enzyme, and the cleavage next to tyrosine residues 73, 76, and 115 was markedly reduced in the acetylated enzyme (5–15% of its value in the native enzyme).

TABLE I: Cleavage of Tyrosyl Peptide Bonds in Reduced and Carboxymethylated Ribonucleases.[a]

Split Bond Next to Tyr No.	Amino Terminal Released	Cleavage (%)	
		RNase	Ac_3-RNase
25	CM-cysteine[b]	45	37
73	Glutamine[c]	47	5
76	Serine	60	9
92	Proline[d]	62	40
97	Lysine[e]	52	44
115	Valine	56	3

[a] In pyridine–acetic acid buffer (pH 3.3), for 45 min at room temperature with 60 equiv of NBS. The new amino terminals were dinitrophenylated with FDNB, and then hydrolyzed, separated, and determined quantitatively by a spectrophotometric assay. [b] Determined as a CM-cysteine sulfone derivative. [c] Determined as a glutamic acid derivative. [d] Determined enzymaticaly with proline iminopeptidase. [e] Corrected for the contribution of the N-terminal lysine.

Discussion

Many studies by other workers using spectrophotometric titrations and chemical modifications of tyrosine residues have revealed at least two classes of tyrosine residues in many proteins. Exposed residues are thought to be at the surface of the protein molecule in direct contact with the solvent and therefore readily accessible to chemical modifications. Buried tyrosyls are considered to be embedded within the interior of the protein, or interacting with other residues in some manner and therefore relatively inaccessible to chemical modifications in the native protein molecule. When the protein native conformation is destroyed by denaturation, all of the tyrosine residues behave "normally." Riordan *et al.* (1965) have introduced the use of *N*-acetylimidazole for the modification of exposed tyrosine residues in proteins. Since then, the method was successfully applied to many proteins in which the number of the exposed and buried tyrosines were determined. However no method for the location of these residues was available, since the *O*-acetyl group in an acetylated tyrosine residue is relatively unstable and is readily hydrolyzed by nucleophiles (Riordan *et al.*, 1965). The peptide bond next to tyrosyl residues in proteins can be cleaved with NBS under acidic conditions (Schmir *et al.*, 1959; Schmir and Cohen, 1961; Wilson and Cohen, 1963a,b). This cleavage reaction cannot distinguish between exposed and buried tyrosine residues since cleavage of these peptide bonds and the oxidation of the cystine residues to cysteic acid residues (Wilson and Cohen, 1963b) result in denaturation of the cleaved protein. This cleavage reaction is however prevented by blocking the phenolic hydroxyl of the tyrosine residue; O-acetylation of this moiety can serve as such a blocking group (Shaltiel and Patchornik, 1961, 1963). We used these findings in order to identify the *nonacetylated* and therefore *cleavable* tyrosine residues in proteins; bovine pancreatic ribonuclease served as the first model. About three out of the six tyrosine residues in native RNase could be acetylated (Shaltiel, 1964; Simpson and Vallee, 1966; Riordan and Vallee, 1967). The other three tyrosine residues could be acetylated only after denaturation of the protein. Riordan and Vallee (1967) have shown that *N*-acetylimidazole is a milder reagent than acetic anhydride, and that only 50% of the tyrosine residues in native RNase could be acetylated by this reagent (Simpson and Vallee, 1966). We confirmed these findings, and found that although nearly quantitative acetylation of the lysine residues in RNase could be obtained with both acetylation reagents (when over 500-fold excess of reagent to protein was used), only about 40–50% acetylation of these residues was obtained when 100-fold excess of *N*-acetylimidazole was used. The same excess of acetic anhydride was sufficient to cause nearly complete acetylation of all the lysine residues in RNase.

Using increasing amounts of *N*-acetylimidazole, intermediate degrees of O-acetylation of tyrosine residues in RNase were obtained. The remaining "free" tyrosine residues in partially acetylated RNase were "titrated" with increasing amounts of NBS. Using this titration method (Schmir *et al.*, 1959), we were able to show that only *five* tyrosine residues were titrated in the nonacetylated enzyme, and the number of the *free* and therefore *titratable* tyrosyl groups were gradually reduced to *two* tyrosine residues in an RNase derivative in which 50% of the tyrosine residues was acetylated (AC_3-RNase). The possibility to titrate only five out of the six tyrosine residues in RNase with NBS, has been explained by Wilson and Cohen (1963b), who found that the peptide bond between tyrosine-25 and cysteic acid-26 is not cleaved by NBS, and this tyrosine residue is not titratable with NBS.

These NBS titration studies clearly showed that three titratable tyrosine residues in the native enzyme were blocked in AC_3-RNase and could no longer be titrated with NBS. Two other tyrosine residues could still be titrated in AC_3-RNase, and the sixth tyrosine residue that could not be titrated in the native enzyme was also untitratable in AC_3-RNase. From these studies it appears that the sixth tyrosine residue (25) is not one of the acetylated tyrosine residues in AC_3-RNase.

Upon cleaving acetylated RNase we found that when reduction and alkylation of the disulfide bonds was necessary, some cleavage (up to 10% of the original cleavage yield in the nonacetylated protein) could be observed even in a totally acetylated protein. This was probably due to some deacetylation of the tyrosine residues during the reduction and alkylation process.

Results clearly show that the cleavage next to tyrosine residues 25 and 97 was slightly affected by the acetylation of RNase, indicating that these residues were not acetylated with *N*-acetylimidazole. Cleavage next to tyrosine-92 was not affected up to an acetylation of 35% of the tyrosine residues in the enzyme. Upon further acetylation, cleavage next to this residue dropped to 65% of its original value in the native enzyme, indicating partial acetylation of this residue. Partial

acetylation of tyrosine-92, at higher concentrations of the acetylation agent, could be caused by slight exposure of this residue due to some conformational changes in the partially acetylated enzyme. The cleavage next to the other three tyrosine residues (73, 76, and 115) was markedly reduced in the acetylated enzyme (only 5–15% of its value in the native enzyme), indicating that these residues were acetylated, and therefore could not be cleaved with NBS. The results we obtained by this method are in excellent agreement with the results obtained by Cha and Scheraga (1963a,b), Donovan (1963), and Woody *et al.* (1966), who located the buried tyrosine residues in RNase by iodination studies. The tyrosine residues that were not iodinated in their studies were not acetylated in our studies (namely residues 25, 92, and 97). Furthermore, tyrosine-92 which could have been partially iodinated by somewhat more drastic iodination conditions, could also be partially acetylated when high concentrations of *N*-acetylimidazole were used. The other two buried tyrosine residues (namely residues 25 and 97) were not modified in both cases. These buried residues were not exposed even when about 80% of the lysine residues of this enzyme were acetylated. The assumption that the three O-acetylated tyrosine residues in RNase were identical with the three iodinated tyrosine residues of Scheraga and coworkers, was previously suggested by Simpson and Vallee (1966) as a result of their side-chain cotton effects studies in RNase. Analysis of the three-dimensional structure of ribonuclease A (Kartha *et al.*, 1967) and of ribonuclease S (Wyckoff *et al.*, 1967) shows that tyrosyls-25, -92, and -97 are the buried tyrosyl residues and tyrosyls-73, -76, and -115 are exposed at the surface of the protein molecule.

The method we have introduced here is a relatively simple one for the location of buried tyrosine residues in proteins. Reduction and alkylation of the disulfide bonds need not be an essential step since the tyrosine-cystine sequence is not very common, and in most cases can be normally cleaved with NBS (Schmir and Cohen, 1963b). The existence of the same tyrosyl sequence more than once is somewhat more troublesome, and more than one cycle of end-group analysis should be employed for that specific sequence. The use of an automatic sequenator could simplify the whole analysis. The cleavage should be carried out in pyridine–acetic acid buffer in order to avoid cleavage of histidyl peptide bonds (Shaltiel and Patchornik, 1963). Tryptophanyl residues, which are also cleaved by NBS (Patchornik *et al.*, 1958, 1960) should be blocked with Koshland's reagent (2-hydroxy-5-nitrobenzyl bromide) prior to cleavage with NBS (Wilchek and Witkop, 1967; Spande *et al.*, 1967).

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