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Structural Studies of Ribonuclease. XX. Acrylonitrile. A Reagent for Blocking the Amino Groups of Lysine Residues in Ribonuclease*

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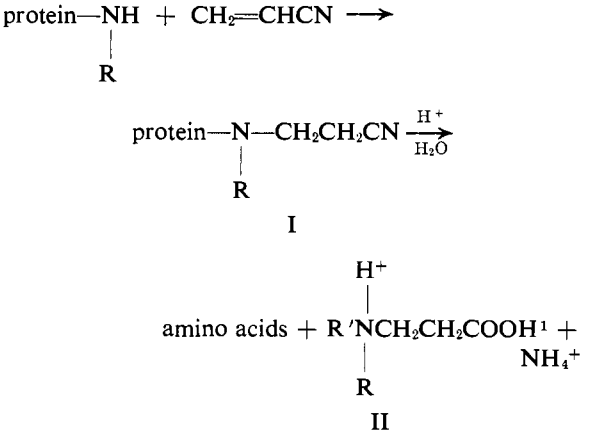
ABSTRACT: The reaction between ribonuclease and acrylonitrile is described. It has been possible to demonstrate that acrylonitrile reacted with the ϵ-amino groups of lysine residues in this protein.

In addition, a derivative was prepared in which all ten lysine residues had reacted with this reagent. Although this derivative was shown to be enzymatically inactive, it did possess physicochemical properties which were similar to those of ribonuclease.

Acrylonitrile has been utilized by Weil and Seibles (1961) to specifically block the thiol groups of reduced β-lactoglobulin and reduced α-lactalbumin, and by Plummer and Hirs (1964) to specifically block the thiol groups of reduced ribonuclease A and reduced ribonuclease B. Although the studies of Weil and Seibles showed that acrylonitrile reacted only with thiol groups, Plummer and Hirs noted a slow reaction with the ϵ-amino groups of lysine residues when the reaction was allowed to proceed above pH 9.5. Subsequently, Kalan *et al.* (1965) reported that the cyanoethylation of β-lactoglobulin and κ-casein resulted in a 25 and 40%, respectively, loss in lysine content. Additional information (McKinney *et al.*, 1950, 1951, 1952) also suggests that the side chains of lysyl and/or histidyl residues could react under certain experimental conditions.

As shown in the accompanying scheme, the reaction between a protein and acrylonitrile is visualized as yielding the cyanoethyl derivatives of active hydrogen compounds, such as those of lysine and histidine residues (I). Since it contains two hydrogens on its primary ϵ-amino group, lysine could react to produce the mono- and/or the dicyanoethyl derivative, while histidine could yield the 1- and/or the 3-cyanoethyl derivative. Subsequent acid hydrolysis (McKinney *et al.*, 1952) would undoubtedly yield the corresponding carboxyethyl compounds (II). These carboxyethyl compounds should chromatograph (on amino acid analysis according to the procedure of Moore *et al.*, 1958, as adapted by Piez and Morris, 1960) at a faster rate than do the parent compounds. Furthermore, since these derivatives are primary α-amino acids, they should produce ninhydrin-positive zones. Therefore, any decrease in the lysine or histidine content of a protein, after acrylonitrile treatment and acid hydrolysis, should be accounted for by the appearance of the respective carboxyethyl derivatives.



This paper reports on the reaction between acrylonitrile and ribonuclease, a protein which contains no thiol groups. It was possible to demonstrate that

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[1] R and R′ would be H and $HOOCCH(NH_3^+)(CH_2)_4$, respectively, if the nitrogen-containing group were lysine.

acrylonitrile reacted with the ε-amino groups of lysine residues and that both mono- and disubstituted derivatives were formed. In addition, a product was obtained which was cyanoethylated at all of the ten lysine residues; the other amino acids in this derivative apparently were not chemically altered. The protein derivative was enzymatically inactive, possessed three abnormal tyrosine residues (as does ribonuclease), and underwent thermal transitions similar to the native molecule.

Experimental Section

Materials. Five-times-crystallized ribonuclease (lot R23 B-55) was purchased from the Sigma Chemical Co. The ribonuclease A fraction was obtained by methods previously described (Rupley and Scheraga, 1963). Acrylonitrile was an Eastman Organic Chemical product and was distilled prior to use. The fraction distilling between 75 and 76° at 748 mm was collected. Poly-L-lysine hydrobromide (degree of polymerization = 866) was purchased from Mann Research Laboratories. Diethylaminoethyl-cellulose (DEAE-cellulose) was obtained from the Brown Co. Sephadex G-25 was purchased from Pharmacia and Amberlite MB-1 from the Rohm and Haas Co. Ninhydrin and hydrindantin were purchased from Pierce Chemical Co. Ribonucleic acid (RNA) and L-lysine monohydrochloride were Nutritional Biochemical Corp. products. All other reagents were either reagent grade or the best grade available.

Methods. PRELIMINARY EXPERIMENTS. Approximately 50 mg of ribonuclease A was dissolved in 10 ml of cold 0.01 M borate buffer, pH 9.2. To the cooled solution (2°) was added sufficient acrylonitrile to make its concentration either 0.1, 0.2, 0.3, or 0.4 M. At 0°, a saturated solution of acrylonitrile in water contains 7.2% acrylonitrile (Davis and Wiedeman, 1945). The solution was stirred in the cold (2°) by means of a Teflon magnetic stirring bar for the desired length of time and then desalted by passage through a 2 × 30 cm column of Sephadex G-25. Ammonium acetate (0.005 M) was employed as the eluting solvent. The protein effluent was pooled and lyophilized, and portions were taken for amino acid analyses.

PREPARATIVE EXPERIMENTS. To a solution of ribonuclease A (*ca.* 250 mg in 50 ml of pH 9.2, 0.01 M borate buffer) at 2° was slowly added 1.35 ml of acrylonitrile. The molar concentration of acrylonitrile was 0.4 M. The solution was placed in a 75-ml capacity polyethylene container; the container was tightly covered and then fixed to a slowly revolving motor which stirred the solution by inverting the container twice per second. At the end of 7 days the reaction mixture was desalted by passage through a 5 × 30 cm column of Sephadex G-25. Again, 0.005 M ammonium acetate was employed as the eluting solvent. The protein effluent was pooled and lyophilized to yield 228 mg of product.

CHROMATOGRAPHY. Preparative chromatography was carried out on a 1.8 × 40 cm DEAE-cellulose column which was equilibrated with 0.005 M borate buffer, pH 9.5. A gradient was produced using a Technicon Autograd as a two-stage apparatus. The first stage contained 300 ml of 0.005 M borate buffer (pH 9.5), and the second contained 300 ml of 0.005 M borate buffer (pH 9.5) which was 0.2 M in sodium chloride. Fractions (6 ml) were collected at a rate of 50 ml/hr and then analyzed for their protein content by measuring the optical density at 280 mμ. The desired fractions were pooled, desalted by passage through a 2 × 40 cm column of Amberlite MB-1, and then lyophilized.

EXPERIMENTS WITH POLY-L-LYSINE HYDROBROMIDE. Approximately 50 mg of poly-L-lysine hydrobromide was dissolved in 20 ml of water, and the pH of the solution was adjusted to pH 9.0 by the addition of 1 M KOH. To the stirred solution was added 0.2 ml of acrylonitrile, and the pH was maintained at 9.0 by adding 1 M KOH from a Radiometer Titrator (TTT1). After 12 hr the precipitate which had begun to form after 3-hr reaction time was dissolved by lowering the pH to 3.0. The solution was dialyzed in the cold (2°) against three changes of 0.001 M HCl and lyophilized to yield 35 mg of cyanoethylated polylysine. A portion of the reaction product was then prepared for amino acid analysis.

TRICYANOETHYLLYSINE. L-Lysine monohydrochloride (9.1 g) was dissolved in 100 ml of 1 N NaOH, and the solution was cooled to 2°. Acrylonitrile (30 ml) was then added over a period of 30 min to the cooled solution. The reaction was allowed to proceed, with stirring, at 2° for 1 day and then at room temperature for 2 days. The solution was neutralized with 4.16 ml of concentrated HCl, whereupon a voluminous precipitate immediately formed. The precipitate was filtered, dried *in vacuo* over phosphorus pentoxide, and then recrystallized from water to yield 3.4 g (22.3%) of colorless material melting at 203–204° dec.

Anal. Calcd for $C_{15}H_{23}N_5O_2$: C, 59.02; H, 7.54; N, 22.95. Found[2]: C, 59.17; H, 7.58; N, 23.04.

The ninhydrin color yield (at 570 mμ), as calculated in leucine equivalents, was 0.03. A portion of this compound (11.85 mg) was prepared for amino acid analysis.

AMINO ACID ANALYSES. Analyses were performed on the Technicon amino acid analyzer. Hydrolysis of the protein samples was carried out by heating in 6 N HCl at 110° for 22 hr in evacuated and sealed ampoules. The amino acid content of each ribonuclease hydrolysate was determined by assuming the theoretical number (15) of aspartic acid residues. The correction factors of Rupley and Scheraga (1963) were employed for those amino acids which undergo decomposition during acid hydrolysis.

RIBONUCLEASE ACTIVITY MEASUREMENTS. Enzymatic assays were carried out in a Beckman Model DU spectrophotometer according to the procedure of Kunitz (1946). RNA in 0.1 M acetate buffer (pH 5.0) was the substrate.

POTENTIOMETRIC TITRATIONS. These measurements

[2] Analysis performed by Schwarzkopf Microanalytical Laboratory, Woodside 77, N.Y.

TABLE I: Amino Acid Composition of Ribonuclease A following Acrylonitrile Treatment.[a]

Amino Acid	Theory	RNAase A (control)	0.1 M (3 days)	0.1 M (7 days)	0.2 M (7 days)	0.3 M (3 days)	0.4 M (7 days)
Aspartic acid[b]	15	15.0	15.0	15.0	15.0	15.0	15.0
Threonine	10	9.9	9.6	10.1	9.8	10.4	10.2
Serine	15	14.9	14.4	14.8	15.0	15.5	14.4
Glutamic acid	12	12.1	11.9	12.2	12.1	11.6	11.5
Proline	4	4.2	4.6	4.3	4.5	4.5	4.3
Glycine	3	2.9	2.9	3.0	3.2	3.2	2.9
Alanine	12	12.2	11.6	11.9	11.8	12.3	11.8
Half-cystine	8	7.8	8.3	8.3	8.4	8.1	8.2
Valine	9	8.5	8.9	8.5	8.7	8.8	8.5
Methionine	4	3.8	3.6	3.5	3.9	3.5	3.6
Isoleucine[c]	3	2.2	2.2	2.4	1.9	2.2	1.9
Leucine	2	1.9	2.0	2.0	2.1	2.0	1.9
Tyrosine	6	5.7	6.0	6.2	6.0	6.1	6.1
Phenylalanine	3	2.7	2.9	3.2	3.1	3.0	3.2
Lysine	10	9.7	6.0	4.0	2.7	2.9	0.4
Histidine	4	3.9	4.2	3.8	4.0	3.8	3.5
Arginine	4	3.8	3.9	3.9	4.1	3.9	3.7

[a] Moles of amino acid/mole of protein. [b] The theoretical number (15) of aspartic acid residues was assumed to be present in each protein hydrolysate. [c] Isoleucine is known not to be completely liberated in a 22-hr acid hydrolysate.

were made in 0.15 M KCl, using a Radiometer Model PHM 4 meter equipped with a G222B glass electrode and a Type K100 calomel reference electrode. The meter was standardized against phthalate, borate, tetroxalate, and mixed phosphate buffers. These buffers were prepared according to the recommendations of Bates (1954). Titrations were performed with approximately 1 M HCl and carbonate-free 1 M KOH. Carbon dioxide was excluded by continuously purging the titration vessel with water-saturated, carbon dioxide free nitrogen.

SPECTRAL STUDIES. Spectrophotometric titrations at 295 mμ were carried out in conjunction with a Beckman DU spectrophotometer. The pH which was measured with a Beckman Model GS pH meter was changed by successive additions of 1 M KOH.

Ultraviolet difference spectral measurements were carried out with a Cary Model 14 recording spectrophotometer according to the procedure of Hermans and Scheraga (1961). The temperature of the reference and sample cells were controlled by circulating water from external baths. The difference spectrum was measured at 286 mμ by maintaining the sample cell at 6° and the reference cell at a temperature which was varied from 6 to 70°. Both cells contained the same solution. Thermal equilibrium was attained by allowing the reference cell to stand at any given temperature for 30 min.

Results

Table I illustrates the effect of acrylonitrile concentration and of reaction time on the amino acid content of ribonuclease. In each case acrylonitrile treatment produced a notable decrease in the lysine content of the protein. It is noteworthy that treatment of ribonuclease A with 0.4 M acrylonitrile at pH 9.2 and at 2° for 7 days yielded a product which was almost deficient in lysine. On the other hand, the concentrations of the remaining amino acids were essentially the same, within experimental error, as those normally observed in a ribonuclease A hydrolysate. It is apparent from these data that acrylonitrile reacted with the ε-amino groups of the lysine residues in ribonuclease. In addition, the histidine content of the cyanoethylated hydrolysates indicated that no detectable reaction had occurred with the imidazole group.

Amino acid chromatography of the cyanoethylated protein hydrolysates produced two ninhydrin-positive zones which were not present in ribonuclease A hydrolysates. One component appeared just prior to and well separated from isoleucine; the second was eluted just after phenylalanine. These two components are undoubtedly the di- and monocarboxyethyl derivatives of lysine. Further evidence for this conclusion was obtained from chromatograms of the cyanoethylated poly-L-lysine hydrolysate. In addition to the expected lysine peak, these chromatograms contained the two components described above. Since the dicarboxyethyl derivative would be expected (on a charge basis) to chromatograph at a faster rate than the monocarboxyethyl derivative, it is assumed that the component eluted just prior to isoleucine is the dicarboxyethyl compound. Kalan *et al.* (1965) have reported that the cyanoethylation of β-lactoglobulin and κ-casein resulted in a decrease in the lysine content of these proteins. In addition, amino acid analyses of the protein hydroly-

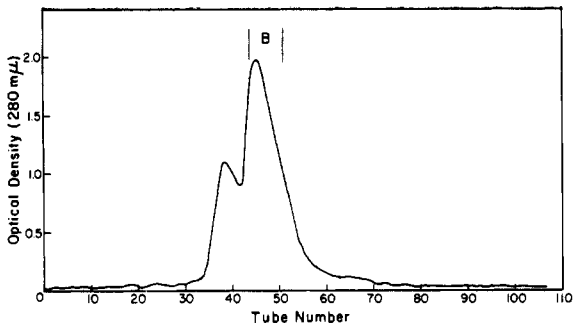

FIGURE 1: Chromatography of 200 mg of ribonuclease which was treated with 0.4 M acrylonitrile for 7 days at pH 9.2 and 2°. Chromatography was carried out on a 1.8 × 40 cm DEAE-cellulose column at pH 9.5 in 0.005 M borate buffer. A two-stage gradient (0–0.2 M in sodium chloride) was used to develop the chromatogram. Fractions (6 ml) were collected at a rate of 50 ml/hr. Those fractions represented by the vertical lines were pooled, desalted, and lyophilized.

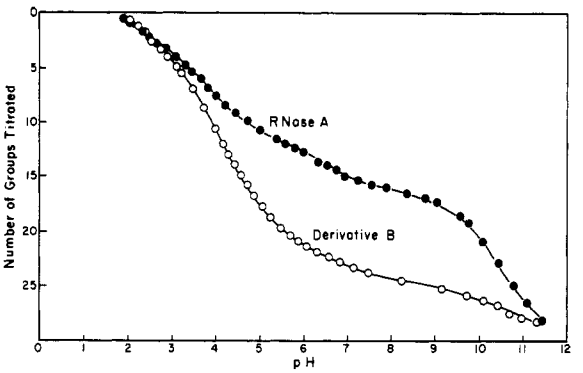

FIGURE 2: Potentiometric titration curves of ribonuclease A (filled circles) and derivative B (open circles) carried out in 0.15 M KCl and at 25°. The positions of the curves relative to one another and to the zero on the ordinate are arbitrary.

sates yielded a new peak which was eluted after phenylalanine. They assumed this peak to be N^{ϵ}-carboxyethyllysine. It is also noteworthy that the ammonia zones in the chromatograms of the cyanoethylated protein hydrolysates were present in high concentrations. This observation should be expected, since cyanoethylation followed by acid hydrolysis would result in ammonium ion formation. Indeed, the increase in the ammonia peak (over that normally found in a ribonuclease hydrolysate) could be used to determine the extent of cyanoethylation. However, we have noted variations in the ammonia concentrations of ribonuclease A hydrolysates; this could be due to differences in the ammonium ion concentration of the buffer systems used for amino acid chromatography or of the 6 N HCl which was used for acid hydrolyses. Therefore, no attempt was made to correlate the extent of cyanoethylation with the increase in the ammonia content.

TABLE II: The Concentration of Lysine, Carboxyethyllysine, and Dicarboxyethyllysine in Hydrolysates of Acrylonitrile-Treated Ribonuclease A.[a]

Concn of Acrylonitrile (M)	Time of Reaction (Days)	Lysine	Carboxyethyllysine	Dicarboxyethyllysine	Total
0.1	1	7.6	1.7	0.6	9.9
0.1	3	6.0	1.5	2.1	9.6
0.1	7	4.0	1.0	4.0	9.0
0.2	7	2.7	0.8	5.4	8.9
0.3	3	2.9	0.8	5.4	9.1
0.3	7	1.2	0.6	7.2	9.0
0.4	7	0.4	0.2	8.2	8.8

[a] Moles of amino acid/mole of ribonuclease.

Table II shows the concentrations of lysine, carboxyethyllysine, and dicarboxyethyllysine obtained from acrylonitrile-treated ribonuclease hydrolysates. The concentrations of the carboxyethyl and dicarboxyethyl compounds were obtained by assuming that their ninhydrin color values were equivalent to that of leucine.[3] This assumption appears to be valid since, in the early stages of the reaction, the *total* lysine content (the sum of lysine, carboxyethyllysine, and dicarboxyethyllysine) approached the theoretical value of ten lysine residues/mole of protein. As the reaction proceeded toward completion the *total* lysine content approached nine residues/mole of protein. It is concluded that the amino-terminal lysine residue of ribonuclease has reacted at both the α-amino and the ϵ-amino groups to form a ninhydrin-negative derivative. Complete cyanoethylation of this residue should cause the *total* lysine content in the acrylonitrile-treated protein hydrolysate to be decreased by one residue; *i.e.*, nine instead of ten residues. Support for this assumption was obtained from studies of the reaction between lysine and acrylonitrile. A derivative was isolated which appeared (from the elemental analysis) to be the tricyanoethyllysine compound. This compound yielded a ninhydrin color value which was only a fraction (3%) of the color yield of leucine. In addition, acid hydrolysis of the cyanoethylated derivative, followed by amino acid chromatography, revealed that the only detectable ninhydrin-positive zone was that ascribed to the ammonia peak.

Isolation of a Fully Cyanoethylated Derivative. The data presented above suggested that a ribonuclease

[3] Slobodian *et al.* (1962) have noted that lysine derivatives blocked at the ϵ-amino group (N^{ϵ}-carbobenzoxylysine and N^{ϵ}-acetyllysine) yielded ninhydrin colors equivalent to leucine.

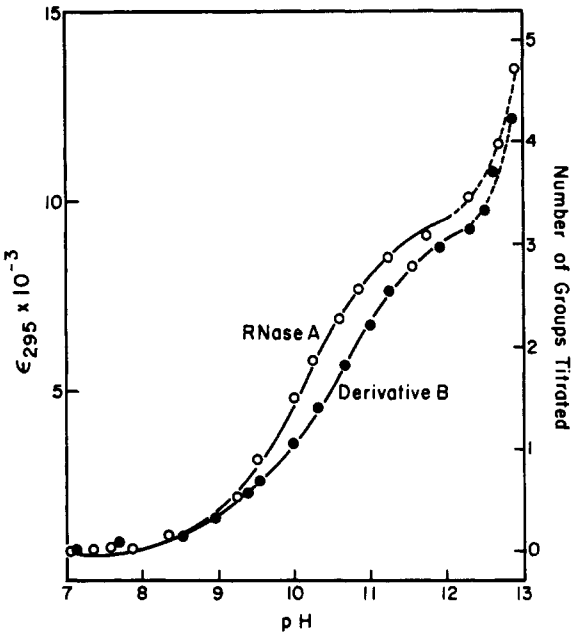

FIGURE 3: Spectrophotometric titrations of ribonuclease A (open circles) and derivative B (filled circles) carried out at 25°. The scale for the number of groups titrated was calculated by assuming an extinction coefficient of 2700 per residue.

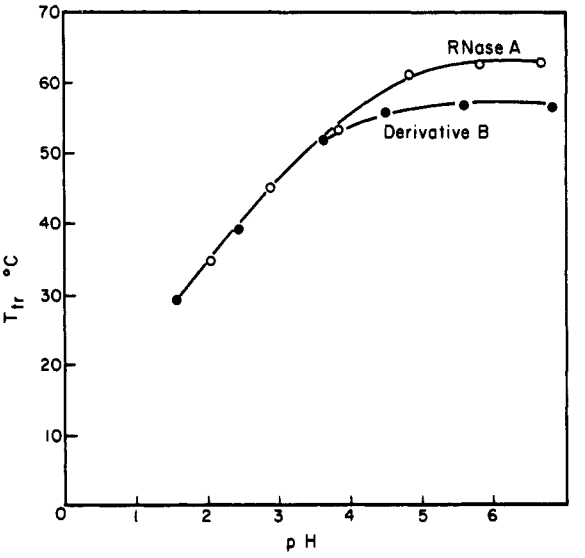

FIGURE 4: Transition temperatures of ribonuclease A (open circles) and component B (filled circles). The transition temperature was taken as the temperature at which one-half the total change in ultraviolet difference extinction coefficient had occurred, between that at low temperature and that at the maximum (high temperature).

derivative which was cyanoethylated at all of the lysine residues could be prepared. Therefore, ribonuclease was treated with acrylonitrile under conditions which would effect complete or nearly complete cyanoethylation.

Figure 1 illustrates the chromatographic separation of ribonuclease (200 mg) which had been treated with 0.4 M acrylonitrile for 7 days at pH 9.2 and 2°. The major component (labeled B in Figure 1) was pooled, desalted by passage through a 2 × 40 cm Amberlite MB-1 column, and then lyophilized to yield 68 mg of protein.

Amino acid analysis of component B indicated that lysine was present in only trace amounts. The concentration of lysine was less than 0.1 residue/mole of protein. The yields of dicarboxyethyllysine and monocarboxyethyllysine were 8.4 and 0.4 residues, respectively, per mole of protein. The yield of histidine was 3.6 residues/mole of protein. The yields of all other amino acids were similar to those obtained from a ribonuclease A hydrolysate. The leading peak of Figure 1 was not isolated; however, it is believed that this derivative (or derivatives) is a ribonuclease component which is not completely cyanoethylated.

Enzymatic Activity of Component B. Component B (the derivative in which all the lysine residues were cyanoethylated) was inactive toward the RNA substrate. This may be due to the cyanoethylation of Lys-41. Hirs (1962) has noted that the dinitrophenylation of Lys-41 leads to the enzymatic inactivation of ribonuclease.

Potentiometric Titrations. Cyanoethylation of an amino group (such as the ϵ-amino group of lysine) should decrease the basicity of this group. McKinney *et al.* (1951) have reported that the pK values of the substituted amino groups in monocyanoethylated amino groups were 2 to 3 pH units lower than the pK values observed for the amino groups in the corresponding amino acids. The pK values of the disubstituted amino groups were approximately 5 pH units lower than the pK values of the unreacted amino groups.

Figure 2 shows a potentiometric titration which was carried out on the fully cyanoethylated ribonuclease derivative (derivative B); also included is a potentiometric titration of ribonuclease A. It is evident from this diagram that the chemically altered lysyl residues titrated at a lower pH than did the ϵ-amino groups of ribonuclease A. Indeed, the majority of the chemically altered amino groups (approximately 8.5 groups) titrated below pH 7.0.

Spectrophotometric Titrations. Figure 3 shows the spectrophotometric titrations which were carried out on ribonuclease A and derivative B. It is noteworthy that derivative B contains three abnormal tyrosyl residues, as does ribonuclease A. The titration of the three normal tyrosyl residues in derivative B occurs over a slightly higher pH range than does the titration of the normal groups in the native molecule. This is probably due to the difference in electrostatic charge between derivative B and ribonuclease A in the pH range (pH 9–11) where the normal tyrosyl residues are titrated.

Thermal Transition Studies. Thermal transition studies performed on derivative B and ribonuclease A are shown in Figure 4. Below pH 3.5 the transition temperatures (T_{tr}) of the two proteins are similar. However, above this pH a notable difference in T_{tr} was observed. The transition temperatures for derivative B (between pH 3.5 and 7.0) are 5–10° lower than those for ribonuclease.

Discussion

Although acrylonitrile has been utilized as a thiol-specific reagent (Weil and Seibles, 1961; Plummer and Hirs, 1964), its use for blocking other functional groups in a protein has not, heretofore, been explored. It is apparent from the data presented above that acrylonitrile reacted (under the experimental conditions described) with the ε-amino groups of the lysyl residues in ribonuclease. It is also noteworthy that acrylonitrile apparently did not react with the imidazole nitrogens of histidine residues.[4] In addition, no detectable reaction with the disulfide cystine residues was observed; thus, this reagent did not detect any disulfide interchange.

It has been possible to employ acrylonitrile in the preparation of a ribonuclease derivative which was cyanoethylated at all ten lysyl residues. The majority of the lysyl residues in this derivative were converted to dicyanoethyllysine residues. Although the cyanoethylated protein was enzymatically inactive toward the RNA substrate, it possessed physicochemical properties similar to those of ribonuclease A. The derivative possessed three abnormal tyrosyl residues, as does ribonuclease A, and underwent thermal transitions, in the acid range, which were similar to those of the native molecule. However, subtle differences were noted from pH 3.5 to 7.0. In this pH range the transition temperatures were 5–10° lower than those observed for ribonuclease. These results indicate that most of the lysyl residues of this protein are to be found near the surface of the molecule, accessible to chemical modification, and that chemical modification of these residues does not grossly alter the conformation of the protein. These conclusions are in agreement with similar ones reported by Klee and Richards (1957) and Cooke *et al.* (1963).

Cyanoethylation of the ε-amino groups of lysyl residues in a protein lowers the pK of these residues. In the case of ribonuclease, where a derivative was formed in which all the lysyl residues were cyanoethylated, the majority of the modified lysyl residues in this derivative titrated below pH 7.0. Therefore, this reagent could be used to study the effect of large changes in electrostatic charge (occurring over a pH range which should not produce protein denaturation) on the conformation of a protein. It was noted previously that the transition temperatures (below pH 3.5) of the fully cyanoethylated protein were similar to those of ribonuclease. From pH 3.5 to 7.0 the transition temperatures of the derivative were notably lower than those of the native protein, thereby indicating that the derivative possessed (in this pH range) a somewhat looser structure than does ribonuclease. It is believed that the looser structure of the derivative between pH 3.5 and 7.0 is due to the large change in electrostatic charge on the protein which occurs in this pH range. If the lower transition temperatures were due to the presence of the cyanoethyl groups, then one would expect the ribonuclease derivative to be less stable than ribonuclease at pH values below 3.5. As was noted previously, this was not the case.

Acrylonitrile is an additional chemical reagent which may be used to block the ε-amino groups of lysyl residues in proteins. Nevertheless, there are disadvantages to its use. First of all, if accessible thiol groups are present in a protein they will undoubtedly react at a very rapid rate. Indeed, since the thiol group is a strong nucleophile, cyanoethylation of the thiol groups would be expected to be much more rapid than the cyanoethylation of ε-amino groups. Second, cyanoethylation of ribonuclease was, under the conditions described in the Experimental Section, quite slow; to effect almost complete cyanoethylation required reaction for 7 days with 0.4 M acrylonitrile. On the other hand, experimental conditions (low temperature and pH 9.2) were used to minimize irreversible protein denaturation. Thus, higher temperature and/or higher pH should bring about a much faster reaction rate. Third, cyanoethylation yields both the mono- and the disubstituted lysine derivatives. However, the protein component (derivative B) which was cyanoethylated at all ε-amino lysyl residues contained (in the main) the disubstituted lysine derivative. This would undoubtedly be the situation (with any protein) if complete cyanoethylation of lysyl residues were to be attempted.

In the case of proteins in which thiol groups are absent, this communication indicates that acrylonitrile can be employed to specifically and completely react with the ε-amino groups of lysyl residues. The extent of cyanoethylation may be determined by amino acid analysis since the reaction products (the carboxyethyl compounds) produce ninhydrin-positive zones.

[4] The histidine content of the fully cyanoethylated derivative (derivative B) was slightly lower than that of ribonuclease. Thus, a small amount of cyanoethylation may have occurred at the imidazole nitrogens. However, no ninhydrin-positive zone(s) which could be ascribed to carboxymethylhistidine was noted in chromatograms of the cyanoethylated ribonuclease hydrolysates.

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Structural Studies of Ribonuclease. XXI. The Reaction between Ribonuclease and a Water-Soluble Carbodiimide*

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ABSTRACT: As a further attempt to modify the carboxyl groups of ribonuclease in an aqueous medium, we report that the reaction between this protein and a water-soluble carbodiimide (at pH 4.5) produces a number of ribonuclease derivatives. Although the exact nature of the reaction product is not known (it is probably either an acylurea or an imide), five derivatives (designated as B, D, F, G, and H) have been isolated and examined. It appears that each of these components differs from ribonuclease in the number of carboxyl groups which have been modified. Peptide analyses and titration data indicate that component B is a derivative which has been modified at one of the eleven carboxyl groups (Asp-53) of ribonuclease. Similar studies on component D indicate that this derivative possesses three modified carboxyl groups (Asp-53, Glu-49, and Glu-111). Component F appears to be a mixture of two derivatives; approximately half of this component is a ribonuclease derivative which has reacted at Asp-53, Glu-49, Glu-111, and Glu-9, while the remaining half has reacted at Asp-53, Glu-49, Glu-111, and Glu-86. Component G has been modified at five of the eleven carboxyl groups (Asp-53, Glu-49, Glu-111, Glu-9, and Glu-86) of the native molecule. In addition to these five carboxyl groups, component H has also been modified at Asp-38. Thermal transition studies and spectrophotometric titration data indicate that each derivative possesses a somewhat looser structure than does ribonuclease. However, components B, D, F, and G appear to contain three abnormal tyrosyl residues, as does the native molecule. On the other hand, component H contains only *two* abnormal tyrosyl residues. Since this derivative differs from component G only in that reaction has occurred at Asp-38, it is concluded that Asp-38 could be involved in a specific tyrosyl–carboxylate interaction believed to be present in ribonuclease.

It has been inferred, from ultraviolet difference spectral data and optical rotation data at low pH, that one or more of the three abnormal tyrosyl residues in ribonuclease (Shugar, 1952; Tanford *et al.*, 1955) are involved in specific tyrosyl–carboxylate interactions (Hermans and Scheraga, 1961). Hermans and Scheraga suggested that these interactions exist in nonpolar regions of the molecule. The existence of specific tyrosyl–carboxylate interactions is further supported by the fact that, in order to fit the experimental potentiometric titration curve with a theoretical curve, it is necessary to assume that some of the carboxyl groups possess a lower pK^0 (intrinsic ionization constant) than do the remainder.

Since the sequence of amino acids in ribonuclease is known, the identification of the abnormal tyrosyl residues as well as the identification of the "buried"[1] carboxyl residues would add valuable information about the conformation of this protein in solution.

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[1] The term "buried" refers to those carboxyl groups which are believed to be involved in specific tyrosyl–carboxylate interactions.