The Role of Lysine in the Action of Bovine Pancreatic Ribonuclease A†

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ABSTRACT: The involvement of lysine residues in the active site of pancreatic ribonuclease has been investigated by assessing (a) the degree of substrate and substrate analogue protection of individual lysine residues against acetylation, and (b) the individual contribution of remaining unacetylated lysine residues to the total catalytic activity of the enzyme. Different substrate analogues (RNA digest, CMP, ATP, and pyrophosphate) were found to give different degrees of protection against acetylation with acetic anhydride. Instead of the expected specific protection of active site lysine residues such as lysine-7 and lysine-41, however, a general decrease in reactivity of all the lysines was observed when the substrate analogues were present during the acetylation. The fraction of enzymatic activity remaining in the protected samples was consistently greater than the fraction of any one lysine remaining unacetylated, and was found to correspond fairly well with the sum of the fractions of unacetylated lysine-7, lysine-41, and a third residue, tentatively assigned as lysine-66. This is consistent with other observations of ribonuclease which suggest that while no lysine residue interacts with substrate and substrate analogues in the formation of the Michaelis–Menten complex, a lysine amino group is required for catalysis. It is proposed that this lysine amino group can be supplied by any one of two or three lysine residues (7, 41, and 66) located close to the substrate binding site.

Several experimental observations clearly implicate lysine ϵ-amino groups as part of the catalytic apparatus of pancreatic ribonuclease, but the total evidence is difficult to interpret in terms of a unified model of ribonuclease structure and function. On the one hand, chemical modification of either Lys-41 (Hirs, 1962; Heinrikson, 1966; Carty and Hirs, 1968; Raetz and Auld, 1972; Dudkin et al., 1975) or Lys-7 (Means and Feeney, 1971; Raetz and Auld, 1972; Dudkin et al., 1975) gives derivatives with 0–5 or 10–30% activity, respectively, while most of the other lysine residues can be modified with little or no effect on the activity (for a review see Barnard, 1969). The observation (Welling et al., 1973; Beintema, J. J. personal communication) that only lysine-7, -41, and -66 are invariant in the sequence of pancreatic ribonuclease from 29 mammalian and marsupial species suggests a key role for these residues in the activity of the enzyme. On the other hand, the x-ray crystallography data, while confirming that lysine-7, -41, and -66 are uniquely located relatively close to and with free access to the active site, strongly suggest that all of these residues are too far away from the site to interact with the substrate. Direct evidence on this point has been obtained with ribonuclease S′ in which Lys-41, as the closest lysine residue, is observed to be out of van der Waals contact with substrate analogues bound in the active site and in which no significant movement of the Lys-41 side chain is observed when substrate or analogues are added to the active site of the crystalline enzyme (Richards and Wyckoff, 1971). In many of the inactive ribonuclease derivatives in which Lys-41 and -7 have been modified, substrate binding has been observed to be unaffected. This is clearly consistent with the conclusions from x-ray crystallography that these residues do not interact with substrates. More recently Carlisle and coworkers (1974) have shown that in ribonuclease A Lys-7 actually is closer to the active site histidine residues (His-12 and -119) than is Lys-41, and further investigations will be required on the possible interactions of Lys-7 with substrate in ribonuclease A.

The current evidence on the role of lysine residues in the active site of ribonuclease thus appears to contain a major conflict in that one set of data strongly implicates lysines as part of the active site, while another set equally strongly suggests that they do not interact with substrate in the active site at all. The present work was undertaken in an attempt to clarify this point of apparent conflict and obtain a better understanding of the mechanism of action of pancreatic ribonuclease.

Materials and Methods

Bovine pancreatic ribonuclease (Sigma, Type 1A) was purified by ion-exchange chromatography on IRC-50 according to the method of Hirs et al. (1953). The enzyme was assayed using either synthetic Cyd-2′:3′-P (Sigma) (Crook et al., 1960) or yeast RNA (Kalnitsky et al., 1959) as substrate. Bovine pancreatic trypsin (Miles-Servac), thermolysine (Calbiochem), and papain (Sigma) were used without further purification; the latter enzyme was activated by incubation with 0.05 M β-mercaptoethanol for 15 min prior to use. [^{14}C]Acetic anhydride (5 mCi/mmol) and [^{3}H]acetic anhydride (50 mCi/mmol) were obtained from New England Nuclear and diluted with unlabeled acetic anhydride to the desired level of radioactivity. The specific radioactivity of each stock acetic anhydride solution was determined as follows. N^{ϵ}-Acetyllysine was prepared by the reaction of the copper complex of lysine with the radioactive anhydride at pH 6 according to the method of Sanger and Neuberger (1943). The product was purified by ion-exchange chromatography on Dowex AG 50-X2 eluted

† From the Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received July 15, 1975.* This work was supported by a U.S. Public Health Service research grant from the National Institutes of Health (GM 15053).

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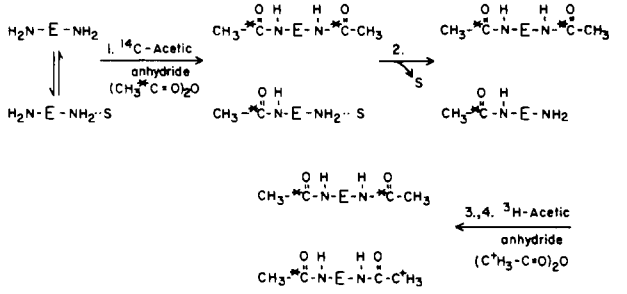
with 0.17 M pyridine acetate buffer (pH 3.8). After lyophilization of the pooled ninhydrin-positive peak, a material was obtained which gave a single ninhydrin-positive, radioactive peak on the long column of the Beckman Model 120C amino acid analyzer. The peak was coincident with authentic N^{ϵ}-acetyllysine and eluted at 84 min (relative to glycine at 91 min). After hydrolysis, the product was converted quantitatively to lysine. The specific radioactivity of the stock solutions of acetic anhydride was determined from the number of counts (determined in a Beckman toluene-Fluoralloy TLA cocktail containing 10% (v/v) Bio-Solv formula BBS-3 solubilizer with a Beckman Model LS-133 liquid scintillation spectrometer) per unit of acetyllysine or lysine (after hydrolysis) as quantified on the amino acid analyzer using phenylalanine as an internal standard to relate the quantities before and after hydrolysis.

Protein concentrations were determined by the method of Sutherland et al. (1949), and free amino groups by titration with trinitrobenzenesulfonate according to Habeeb (1966). All chemicals used were commercial samples of the highest available purity.

Experimental Plan and Methods. The experiments were designed to explore two features of the acetylation of ribonuclease, the effect of certain substrates and substrate analogues as protective agents for specific lysine residues, and the relationship between enzyme activity and the acetylation of specific lysine residues. The general experimental design involved a double isotope labeling technique derived from the method used by Kaplan et al. (1971) to determine the pK_a values of individual lysine amino groups in chymotrypsin. The experimental procedure is illustrated in Scheme I, and can be outlined as follows. (1) Reaction of

Scheme I



ribonuclease with [^{14}C]acetic anhydride in the absence of or in the presence of substrate and substrate analogues, under conditions predetermined to give essentially complete acetylation in the absence of protective agents. (2) Purification of the acetylated enzyme derivatives by (repeated if necessary) gel filtration. Determination of the extent of acetylation by both ^{14}C content and by disappearance of free amino groups. If discrepancy suggests acetylation of tyrosines, removal of acetate from the *O*-acetyltyrosine groups by treatment with hydroxylamine must be carried out. Determination of enzymatic activity and catalytic properties of the purified acetylribonuclease derivatives. (3) Acetylation with a large excess of [^{3}H]acetic anhydride under conditions which will give exhaustive acetylation. (4) Purification of the product by (repeated) gel filtration, removal of *O*-acetyl groups by treatment with hydroxylamine, and careful quantitative determination of ^{14}C and ^{3}H content of each derivative. (The ^{3}H content should be a precise measure of the free amino groups present at step 2 and should agree with the trinitrobenzenesulfonate titration at that stage.)

Subsequent protease digestion of performic acid oxidized, acetylated protein derivatives, should permit isolation of peptides containing individual lysine residues. Determination of the ratio [^{3}H]/([^{3}H] + [^{14}C]) for each separate peptide should in turn give a direct measure of the percent of a particular lysine residue which was present as free lysine in the derivative characterized under (2).

This general procedure should give a measure of the relative degree to which a given lysine residue (1) is protected by substrate analogues against acetylation and (2) contributes to the activity of the enzyme.

Results

Preliminary Studies. Three groups of experimental variables required assessment prior to the experimental approach outlined above: the general conditions for the acetylation, the nature of the protective agents, and the total effect of the hydroxylamine treatment. The detailed data obtained in the assessment of these variables will not be given, but the reasoning and the results that were the basis for the final experimental design are summarized below.

The acetylation of ribonuclease with an excess of acetic anhydride gave an optimal rate of disappearance of amino groups at pH 8.7. Considering both the potential nonspecific electrostatic interaction between lysine amino groups and anionic protective agents and the possible side reactions of acetic anhydride with tyrosine, it was deemed desirable to carry out the acetylation in the presence of high concentrations of acetate (Vallee and Riordan, 1964). When ribonuclease was assayed in the presence of 1 M sodium acetate in 0.5 M borate buffer (pH 8.7), the activity was 90–95% of that observed at low salt concentration, and the above conditions were consequently selected as standard reaction conditions. The acetylations were carried out at 4° and this buffer is actually supersaturated with respect to borate at this temperature. The precipitate formed at 4° was generally removed, but could also be left in the reaction mixture without any effect.

The selection of protective agents (substrates and substrate analogues) was based on the desire to correlate the data with those obtained in x-ray crystallography and at the same time use compounds known to be strong competitive inhibitors of the enzyme. CMP (mixed 2′- and 3′-phosphate) was selected as the most obvious substrate analogue satisfying both of these criteria. In addition, ATP and inorganic pyrophosphate were included as good competitive inhibitors and finally yeast RNA as an actual substrate. In the latter case it is unlikely that any intact RNA remains in the reaction mixture during the acetylation. With the high level of enzyme, hydrolysis should be very fast, and although we have no data on this point, we assume that the RNA that interacted with the enzyme was a mixture of oligonucleotides produced through the enzyme-catalyzed hydrolysis.

It is essential in these experiments that only lysine residues are acetylated. Since radioactivity is the only measure of the extent of lysine modification, other acetylation products would confound the interpretation of the results. In spite of the fact that the high acetate concentration should prevent tyrosine acetylation (Vallee and Riordan, 1964), it was decided that all acetylated derivatives should be treated with hydroxylamine under conditions known to cleave *O*-acetyltyrosine (Hauser and Renfrow, 1937). The treatment

Table I: Reaction Conditions for the Acetylation of Ribonuclease.[a]

Expt	Protective Agent (Substrate or Substrate Analogue)	Concn of Protective Agent (mM)	Concn of Acetic Anhydride: First Acetylation[b] (mM)	Concn of Acetic Anhydride: Second Acetylation (mM)
1	None		7.5	460
2	None		10.4	460
2	Yeast RNA	58[c]	10.4	460
1	CMP (K_I = 2 mM)[d]	217	7.5	460
2	CMP (K_I = 2 mM)[d]	235	10.4	460
1	ATP (K_I = 4 mM)[d]	230	7.5	460
1	Pyrophosphate (K_I = 5 mM)[e]	126	7.5	460

[a] Each reaction mixture contained 100 mg of ribonuclease in 20 ml of 0.5 M sodium borate–1 M sodium acetate buffer (pH 8.7) corresponding to a ribonuclease concentration of 0.4 mM. [b] In experiment 1 the acetic anhydride was added in three aliquots. In experiment 2 the acetic anhydride was added in a single aliquot. [c] Concentration of RNA expressed in terms of millimoles of nucleotide monomer per liter. [d] Ukita et al. (1961). K_I values determined at pH 7.6 in 0.1 M salt. [e] Anderson et al. (1968). K_I value determined at pH 7.5 in 0.2 M salt.

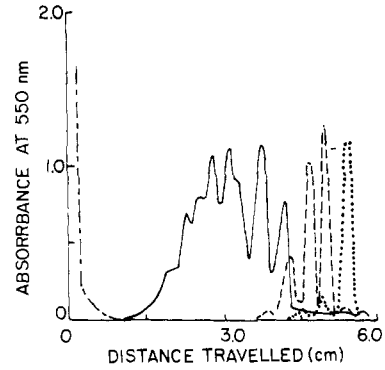


FIGURE 1: Densitometer scans of disc gel patterns of ribonuclease and acetylated ribonuclease derivatives. (- · -) ribonuclease; (—) ribonuclease acetylated in the presence of RNA; (- - -) ribonuclease acetylated in the presence of CMP; (· · ·) ribonuclease acetylated in the absence of protective agents. The electrophoresis was carried out with 5 μg of protein in the pH 4.3 system described by Reisfield et al. (1962), using a Canalco Model 12 electrophoresis apparatus with 5 × 80 mm tubes containing running gel only.

of native ribonuclease, and the proper *N*- and *O*-acetyl derivatives with 1 M NH_2OH in 1 mM EDTA (to prevent the side reactions reported by Lin (1972)) adjusted to pH 8, for 20 min at 27°, was found to affect neither enzymatic activity nor the *N*-acetyllysine derivatives, while *O*-acetyltyrosine derivatives were quantitatively cleaved. Based on these preliminary investigations the experimental conditions outlined in Table I were established.

Acetylation of Ribonuclease in the Presence and Absence of Protective Agents. Ribonuclease was acetylated (Fraenkel-Conrat, 1957) in two separate sets of experiments with different protective agents and with a level of [^{14}C]acetic anhydride which gave essentially complete inactivation of the unprotected enzyme. The acetic anhydride was added as an acetone solution, and the reaction mixture was generally left with stirring overnight, although the actual acetylation was completed within the first few minutes. Each reaction mixture was subjected to purification by gel filtration on Sephadex G-25 to remove protective agents, free radioactive acetate, and other low molecular weight compounds. In the case of the RNA-protected sample the purification required an additional ion-exchange chromatography step after the gel filtration. When the mixture of acetylated enzyme and RNA was subjected to chromatography on a Dowex 1-X4 column, equilibrated, and eluted with 0.1 mM HCl, the enzyme was quantitatively recovered in the second column volume, while all the RNA was bound, and could be eluted with 0.5 M KCl. All the radioactivity was associated with the enzyme peak, demonstrating that no acetylation of RNA had taken place.

The purified enzyme fractions were lyophilized and characterized with respect to enzymatic activity, including the kinetic parameters, free amino groups (determined directly by titration with trinitrobenzenesulfonate, or by the difference between total lysine and the amount of acetate incorporated (Table II)), and electrophoretic heterogeneity (Figure 1). It is clear from these results that RNA is by far the best protective agent, in terms of minimizing both the extent of lysine acetylation and loss of activity. The loss of activity appears to be directly related to loss of catalytic capability, the K_m of the modified proteins being essentially unaltered. As expected the product of each acetylation reaction is a mixture of protein derivatives with different extent of acetylation. Only in the unprotected sample is there one primary derivative, presumably fully acetylated ribonuclease (11 acetyl groups). Judging from the distance of the individual peaks from the top of the gel (native ribonuclease) it appears that the CMP-protected sample consists of a mixture of derivatives containing 8, 9, and 10 acetyl groups, and the RNA-protected sample a mixture of derivatives containing (3), 4, 5, 6, 7, and 8 acetyl groups (Figure 1).

The bulk of the purified [^{14}C]acetyl protein derivatives were next acetylated with a large excess of [^{3}H]acetic anhydride (1150 mol/mol of enzyme) under the same conditions as those used in the first acetylation, but in the absence of protective agents. The fully acetylated samples were subjected to gel filtration; the collected protein peaks were treated with hydroxylamine and, after a second purification by gel filtration, were characterized as before. Gel electrophoresis gave a single peak corresponding to the fastest moving derivative in Figure 1, each derivative was completely void of enzymatic activity and the [^{3}H]acetate incorporation (Table II) corresponded well with the number of free amino groups determined after the first acetylation. The total acetylation based on both ^{14}C and ^{3}H incorporation within experimental errors gave 11 groups acetylated in each derivative. At this stage, therefore, a set of chemically identical derivatives, differing only in the amount of ^{14}C and ^{3}H content, has been produced. Degradation of these identical derivatives should permit an assessment of the involvement of the individual lysine residues in the first acetylation reaction.

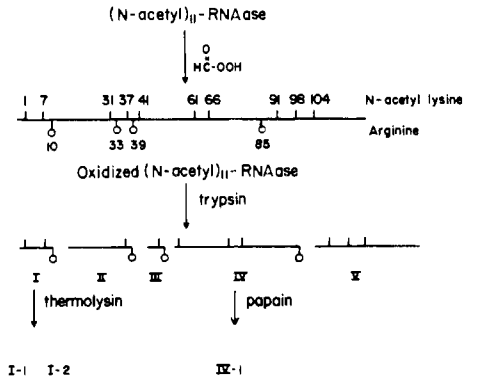
Degradation of N-Acetyl Ribonuclease and Determination of Radioactivity in Individual Fractions. The procedures chosen for the degradation are outlined in Scheme II, and the fractionation of the various peptide mixtures is illustrated in Figure 2. The five tryptic peptides obtained from the oxidized acetyl ribonuclease were readily separated by ion-exchange chromatography on phosphocellulose (Chin and Wold, 1971), and the radioactive peaks (Figure 2A) were identified by amino acid analysis (Table III). Fraction T-1, corresponding to tryptic peptide IV containing lysine residues 41, 61, and 66, was further digested with

Table II: Properties of the Ribonuclease Derivatives Obtained after the First Acetylation and after the Second Acetylation.[a]

(Identified by) Protective Agent	Properties after First Acetylation [^{14}C]						Properties after Second Acetylation [^{3}H]	
	Enzymatic Activity				Acetylation Data		Acetylation Data	
	% Activity							
	Cyd-2′:3′-P	RNA	K_m (mM)	V_{max} (rel)	Free Amino Groups[b] (moles/mole)	^{14}C Incorp (moles/mole)	^{3}H Incorp[c] (moles/mole)	^{14}C + ^{3}H Incorp (moles/mole)
None Expt 1	0	2 ± 2			0.0 ± 0.5	9.7 ± 0.6	0.9 ± 0.1	10.6 ± 0.7
None Expt 2	0				0.0 ± 0.3	10.9 ± 1.2	1.6 ± 0.1	12.5 ± 1.3
RNA Expt 2	102 ± 2	72 ± 27	2.8 ± 1.4	1.2	5.7 ± 0.6	7.0 ± 1.1	4.0 ± 0.1	11.0 ± 1.2
CMP Expt 1	34 ± 2	12 ± 3	2.8 ± 1.3	0.15	1.0 ± 0.5	9.2 ± 0.6	1.3 ± 0.6	10.5 ± 0.7
CMP Expt 2	16 ± 2				1.2 ± 0.5	9.8 ± 1.1	2.1 ± 0.1	11.9 ± 1.2
ATP Expt 1	6 ± 2	8 ± 5	1.9 ± 1.3	0.07	1.3 ± 0.5	8.4 ± 0.6	1.8 ± 0.1	10.2 ± 0.7
Pyrophosphate Expt 1	2 ± 2	5 ± 3	3.9 ± 1.2	0.06	1.5 ± 0.5	9.4 ± 0.6	1.9 ± 0.1	11.3 ± 0.7
Native control	100 ± 2	100 ± 19	2.8 ± 1.3	1.0	11 ± 0.5			

[a] The major source of error in all measurements was the protein concentration determination. The uncertainties reported for each set of values represent the range of observations for a minimum of three replicates together with the standard error in protein concentration determination. [b] Trinitrobenzenesulfonate titration values. [c] The values in this column are maximum levels, since traces of [^{3}H]acetate are likely to have been carried along as contaminants. Subsequent degradation and purification of individual peptides gave a slight reduction in ^{3}H content in some of the samples.

Scheme II: Degradation of Acetylated Ribonuclease.



papain to yield the fractions shown in Figure 2B. The first of these, fraction P-1, contained only lysine; P-3, lysine and aspartate; and the composition of fraction P-4 corresponded to residues 40–45 (Table III). Thus lysine-41 is unequivocally identified, whereas lysine-61 and -66 cannot be distinguished since both of these are adjacent to an asparagine residue. Fraction T-3 corresponding to tryptic peptide I, containing lysine residues 1 and 7, was further digested with thermolysine to give two radioactive fractions (Figure 2C) readily identified as the peptides containing each of the two lysine residues. Attempts to fragment fraction T-2, corresponding to the carboxyl-terminal tryptic peptide V, containing lysine residues 91, 98, and 104, into peptides which would distinguish the three lysine residues were unuccessful. The ratios of [^{3}H]- to [^{3}H + ^{14}C]acetate in individual lysine residues and in peptides are summarized in Table IV.

It is clear that the accumulated errors in the measurements are quite high. Especially disconcerting is the significant background of ^{3}H counts in the unprotected samples, in which no free amino groups could be detected by direct titration with trinitrobenzenesulfonate. It was considered to treat this background as an error inherent in the methods used and subtract these values from the values obtained for the protected samples. Since the actual reason for the relatively high background is not understood, such a correction was not carried out, however. As a consequence, the lower levels of ^{3}H contents (pyrophosphate- and ATP-protected samples) have too large an uncertainty associated with them to permit reasonable interpretation. For the other samples, however, the background becomes a relatively insignificant fraction of the total count. It is important to bear in mind that the value for lysine-1 represents the average of both the α- and ϵ-amino groups of the amino-terminal lysine. Since the ^{3}H content of lysine-1 is consistently lower than that of the others, it is reasonable to assume that the more acidic α-amino group was essentially completely acetylated in all the derivatives and that most of the observed ^{3}H was contributed by the ϵ-amino group. If this assumption is correct, the values for lysine-1 in Table IV should be multiplied by 2 to be directly comparable to the other lysine residues in which only the ϵ-amino groups could be acetylated.

Discussion and Conclusions

Although the attempts to assay all of the individual lysine residues for ^{3}H and ^{14}C content were unsuccessful, the results in Table IV still permit several conclusions to be made. The first of these conclusions concerns the specific interaction of any one lysine residue with substrate analogues. The assumption is that such interaction would lead to a decreased reactivity of the lysine involved, and is based on a large number of cases in which protection against chemical modification of a specific residue is affected by coenzymes and substrates. In the case of ribonuclease there does not appear to be any one lysine residue which is specifically protected. Although the different protective agents definitely provided protection against the acetylation, the protection extends to all the lysine residues with little or no selection. It is difficult to explain this broad general protection, since if there is no interaction of lysines with the protective agents, it would be most reasonable to expect the same extent of reaction in all samples. The possible explanation that the acetic anhydride concentration was decreased through direct reaction with the protective agents appears to be ruled out by the complete absence of radioactivity in the RNA fraction isolated by ion-exchange chromatography of the RNA-protected reaction mixtures. It therefore appears that the conclusion must be that although no single lysine residue interacts strongly with substrate analogues, the addition of the analogues to the enzyme reduces the reactivity of all the lysine residues. A similar broad effect of ligand

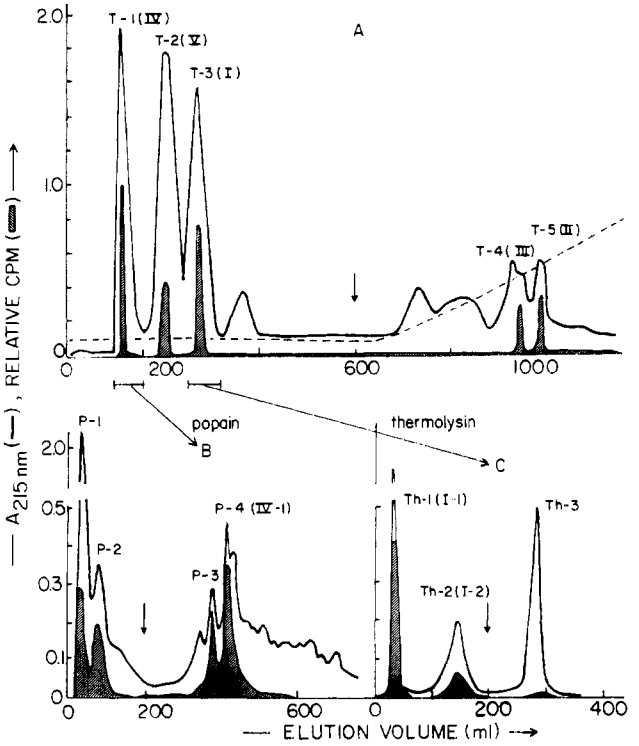


FIGURE 2: Peptide fractionation by ion-exchange chromatography. In A and C the peptide mixture in 0.025 *N* H_3PO_4 (pH 2.4) was applied to a phosphocellulose column equilibrated with the same H_3PO_4 solution. After elution with the same equilibration solution a linear gradient of 0–0.2 M KCl was applied. The vertical arrow indicates the start of the gradient and the dotted line in A illustrates the linear change in conductivity of the eluate. In B the peptide mixture in 5 mM sodium borate buffer (pH 9) was applied to a TEAE-cellulose column equilibrated with the same buffer. After initial elution with the starting buffer, a linear gradient of 5–300 mM sodium borate was applied. The eluate was monitored for absorbance at 215 nm and for radioactivity. Some minor 215-nm absorbing peaks containing no amino acids were observed; these have been left without designation. One radioactive peak (designated P-2 in B) was also devoid of amino acids. (A) Separation of peptides produced by trypsin digestion of oxidized acetyl$_{11}$-ribonuclease. The roman numerals indicate the sequence of peptides in the primary structure of the enzyme. (B) Separation of peptides produced by papain digestion of tryptic peptide T-1 (IV). (C) Separation of peptides produced by thermolysin digestion of tryptic peptide T-3 (I). Although only Th-1 and Th-2 are relevent to this paper it should be noted that Th-3 accounts for the carboxyl terminal end (Phe-Glu-Arg) of the decapeptide.

binding has been observed in the case of CO-binding to hemoglobin (Benson et al., 1973). In that case CO binding caused a shift of the spectrum of rates of hydrogen exchange to more rapid rates, suggesting that large conformational changes are associated with the ligand binding. Similarly, the addition of *N*-acetyl-D-glucosamine to egg white lysozyme caused a significant decrease in the exchange rate of all exchangeable hydrogens (Wickett et al., 1974). In both of these cases the influence of the bound ligand is propagated through the entire protein, and in analogy it appears reasonable to propose that the general reduction in reactivity of lysine residues in the presence of substrate analogues in ribonuclease reflects a conformational change which involves the entire molecule. The lack of specific protection of lysine-41 or -7 must be considered to be consistent with the conclusions derived from x-ray crystallography, that the proposed active site lysines are out of contact with the substrate analogues.

This does not mean, however, that lysine residues could

Table III: Characterization of Individual Peptides by Amino Acid Analysis.

	Tryptic Peptides (Figure 2A)										Papain Peptides (Figure 2B)				Thermolysine Peptides (Figure 2C)			
	Found for Peptide T-1	Calcd for IV (Residues 40–85)	Found for Peptide T-2	Calcd for V (Residues 86–124)	Found for Peptide T-3	Calcd for I (Residues 1–10)	Found for Peptide T-4	Calcd for III (Residues 34–39)	Found for Peptide T-5	Calcd for II (Residues 11–33)	Found for Peptide P-3	Calcd for IV-2 (Residues 61–62 or 66–67)	Found for Peptide P-4	Calcd for IV-1 (Residues 40–44)	Found for Peptide Th-1	Calcd for 1-1 (Residues 1–3)	Found for Peptide Th-2	Calcd for 1-2 (Residues 4–7)
Cys[a]	5.0	5	2.0	2	0.2		0.1		1.0	1	0.3		1.0	1	0.0		0.0	
Asp	5.9	6	3.9	4	0.5		2.0	2	3.1	3	1.2	1	1.2	1	0.1		0.2	
Thr	3.6	4	2.8	3	1.1	1	1.0	1	1.0	1	0.3		0.4		0.7	1	0.5	
Ser	4.4	5	2.9	3	0.4		0.1		6.7	7	0.1		0.4		0.1		0.2	
Glu	5.9	5	3.3	3	2.4	2	0.1		2.3	2	0.1		0.3		0.9	1	0.1	
Pro	0.9	1	2.9	3	0.0		0.0		0.0		0.0		0.9	1	0.0		0.0	
Gly	1.1	1	1.7	2	0.1		0.1		0.1		0.1		0.2		0.0		0.0	
Ala	3.1	3	3.6	4	3.0	3	0.1		1.8	2	0.2		0.3		0.0		2.8	3
Val	4.9	5	3.7	4	0.2		0.1		0.1		0.3		0.9	1	0.0		0.0	
Met[a]	0.9	1	0.1	0	0.2		0.1		2.9	3	0.0		0.1		0.0		0.0	
Ile	0.7	1	1.2	2	0.1		0.1		0.1		0.1		0.2		0.0		0.0	
Leu	0.9	1	0.1	0	0.1		1.2	1	0.1		0.3		0.1		0.0		0.0	
Tyr	1.9	2	1.9	3	0.1		0.1		0.8	1	0.0		0.0		0.0		0.0	
Phe	1.0	1	1.0	1	1.1	1	0.1		0.1		0.0		0.1		0.0		0.0	
Lys	*3.0*[b]	3	*3.0*	3	*2.0*	2	*1.0*	1	*1.0*	1	*1.0*	1	*1.0*	1	*1.0*	1	*1.0*	1
His	0.8	1	1.6	2	0.1		0.1	1	1.0	1	0.0		0.1		0.0		0.0	
Arg	0.7	1	0.1	0	0.9	1	1.0	1	0.9	1	0.0		0.1		0.0		0.0	

[a] Cysteine and methionine were analyzed as the oxidized products, cysteic acid and methionine sulfone. [b] The amino acid analyses were calculated relative to lysine in all cases.

Table IV: Percentage of Unacetylated Lysine after the First Acetylation Measured as Percentage ^3H of Total $^3H + {}^{14}C$ in Different Peptides.[a]

Sample (Identified by Protective Agent)	% Activity in original Derivative (Cyd-2′:3′-P)	% ^3H in Individual Peptides							
		T-3 (Lys-1,-7)	T-1 (Lys-41, -61, -66)	T-2 (Lys-91, -98, -104)	T-4 (Lys-37)	T-5 (Lys-31)	P-4 (Lys-41)	Th-1 (Lys-1)	Th-2 (Lys-7)
None 1	0	2.7 ± 0.1	5.0 ± 0.1	3.0 ± 0.1	2.9 ± 0.1	3.1 ± 0.1	2.9 ± 0.1	3.2 ± 0.1	2.3 ± 0.1
None 2	0	2.4 ± 0.1	8.4 ± 0.2	7.7 ± 0.3	2.2 ± 0.1	3.4 ± 0.2	3.4 ± 0.2	0.9 ± 0.1	0.7 ± 0.1
Pyrophosphate	2	10.3 ± 1.5	11.3 ± 0.9	11.7 ± 1.5	11.1 ± 0.7	11.1 ± 0.9	4.2 ± 0.1	4.4 ± 0.2	4.0 ± 0.2
ATP	6	7.7 ± 1.5	10.3 ± 1.5	9.3 ± 0.7	8.0 ± 0.7	8.0 ± 0.7	7.9 ± 0.1	1.7 ± 0.1	2.5 ± 0.1
CMP 2	16	4.6 ± 0.1	12.0 ± 0.2	11.8 ± 0.7	7.1 ± 0.1	14.7 ± 0.3	8.3 ± 0.5	2.7 ± 0.1	6.2 ± 0.1
CMP 1	34	7.7 ± 0.9	14.0 ± 1.5	8.6 ± 0.7	9.5 ± 0.7	7.7 ± 0.9	14.7 ± 0.2	10.5 ± 0.7	10.5 ± 0.6
RNA 2	102	24.3 ± 0.4	33.0 ± 1.2	32.6 ± 1.1	32.0 ± 1.1	29.0 ± 1.8	29.2 ± 0.3	12.8 ± 0.9	45.0 ± 1.6

[a] Peptide concentrations were determined from amino acid analyses. The error in each determination was obtained from

$$dP = \left(\frac{([d^3H][^3H + {}^{14}C] - [^3H][d^3H + d^{14}C])}{[^3H + {}^{14}C]^2} \right) 100$$

where P, $[^3H]$, and $[^{14}C]$ represent "protection" $(100[^3H]/[^3H + {}^{14}C])$, average cpm of $[^3H]$, and average cpm of $[^{14}C]$, respectively, and dP, $[d^3H]$, and $[d^{14}C]$ are, in order, the calculated error (given in the table), the standard deviation in $[^3H]$ counts, and the standard deviation in $[^{14}C]$ counts. dP Rarely exceeds 10%.

not be involved in the catalytic mechanism of ribonuclease. Assuming that RNA was completely digested to products by the time the acetic anhydride was added, all the reaction mixtures contained static complexes of enzyme and substrate analogues with no catalysis taking place, and the results cannot be strictly interpreted in terms of the role of lysines in the dynamic action of the enzyme.

The other question of interest, whether a single lysine residue is specifically required for activity, can also be answered by the results in Table IV. If catalysis requires participation of a specific lysine residue, the percentage of activity of any one derivative could never exceed the percentage of that residue which remained free after the first acetylation. In other words, if lysine-41 were required, a derivative containing 15% of its lysine-41 unacetylated could have no more than 15% of native activity. Due to the distinct possibility that extensive acetylation could lead to a general denaturation the expected activity for the above example could well be less than the maximum 15%. Such a denaturation may well be what was observed for the low activity samples (pyrophosphate and ATP protection). For all the derivatives with more than 10% activity remaining, however, each derivative has much higher activity than can be accounted for by any one lysine residue. The possibility that the high activity is derived from superactive acetylated species cannot be completely eliminated, but the fact that derivatives with low and intermediate acetyl content never showed activity significantly higher than the unreacted control makes this explanation implausible. This leaves only two possible explanations for this finding and both exclude the possibility that a single lysine residue is uniquely required for activity. The first explanation is that there is no lysine involved in the enzyme activity and that the loss of activity simply reflects general denaturation of the enzyme. The second explanation is that the catalytic activity of the enzyme depends on the participation of a lysine residue, but that more than one residue can satisfy the requirement. Although we have no experimental evidence to exclude the first explanation, the second explanation appears more satisfactory in explaining all the data on chemical modification of lysines in ribonuclease. An examination of a model of the ribonuclease molecule reveals that four lysine residues have direct access to the active site, the other six are shielded from the active site by folds of the polypeptide chain. The four lysines are residues 7, 41, 66, and 104, and according to the measurements of Richards and Wyckoff (1971) the distances of the four charged amino groups from a phosphate group in the active site of ribonucleases S are 8, 6.5, 11, and 19 Å, respectively. The recent work by Carlisle and coworkers (1974) demonstrates that lysine-7 is considerably closer to the active site of ribonuclease A, the form of the enzyme studied in this work, and it thus appears that lysine-7 and -41 and perhaps also -66 are close enough to the active site to provide a positive charge in the vicinity of the catalytic action. This model is quite satisfactory in explaining the data in Table IV. Using an estimated value for lysine-66 acetylation (assuming -61 and -66 to be acetylated to the same extent), the activity of the different derivatives can be accounted for quite well by the sum of free lysine-7, -41, and -66, suggesting that either one of the three residues can provide the acidic function required in the reaction. If this model is correct, it is suggested that the 0–5% catalytic activity observed with the derivatives containing dinitrophenyllysine-41 (Hirs, 1962; Fung and Doscher, 1971), sulfonyloxynitrophenyllysine-41 (Carty and Hirs, 1968), and

pyridoxyllysine-41 (Raetz and Auld, 1972; Dudkin et al., 1975) and the 10–30% activity observed with pyridoxyllysine-7 derivatives (Raetz and Auld, 1971; Dudkin et al., 1975) must to a great extent be due to steric effects of the relatively large and bulky reagent groups. The literature is ambiguous on this point. Allewell et al. (1973) observed a 3 Å displacement of the ϵ-NH_2 group of Lys-41 after dinitrophenylation, but concluded that the group was well separated from the substrate binding site. Dudkin et al. (1975) on the other hand have concluded that the pyridoxyl group in the Lys-41 derivative is located directly in the active site. Thus both steric hindrance and conformational distortion may have to be invoked to explain the loss of activity. The considerably smaller acetyl groups would presumably give a minimum of steric blocking and distortion, and full activity, corresponding to the sum of free lysine-7, -41, and -66, could be observed.

This model provides a reasonably consistent picture of all the observations made on ribonuclease, and satisfactorily explains the apparent conflict between the observations made on the enzyme in solution and in the crystalline state. In the static complex between substrate analogues and enzyme there is no role for lysine residues, and consequently no interaction between the substrate analogue and any one lysine residue is observed. When an actual substrate is situated in the active site, it has been proposed by several workers that catalysis requires a transition state in which a pentacoordinated phosphate must be stabilized through the interaction of one of the oxygens with a positively charged residue (Witzel, 1963; Wang, 1968; Usher, 1969; Roberts et al., 1969). Thus it appears that a lysine is required for catalysis, but any one of two or three lysines can satisfy the requirement. It is interesting indeed that the comparative sequence data compiled by Welling et al. (1973) have established lysine-7, -41, and -66 to be invariant in a large number of ribonucleases.

Acknowledgment

The authors are grateful to Mr. Christopher C. Q. Chin for his contribution to this work of the thermolysin digestion of tryptic peptide I and of many helpful discussions and suggestions.

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