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Cross-Linking of Bovine Pancreatic Ribonuclease A with Dimethyl Adipimidate*

Fred C. Hartman† and Finn Wold‡

ABSTRACT: Modification of bovine pancreatic ribonuclease A with [^{14}C]dimethyl adipimidate, a bifunctional imido ester, resulted in the cross-linking of lysine ϵ-amino groups with a concomitant increase in enzymic activity. Dimers and higher molecular weight derivatives arising from intermolecular reactions were separated from intramolecularly cross-linked monomeric species by gel filtration.

The isolated monomeric components were characterized in terms of molecular weight homogeneity, the extent and specificity of the reagent incorporation, and enzymic activity. A monomeric derivative with a specific activity of 160, compared to 100 for the native enzyme, was degraded by performic acid oxidation and trypsin digestion, and the tryptic peptides were fractionated by preparative paper electrophoresis and chromatography. Two cross-linked peptides, representing at least 50% of the total cross-links in the derivative, were isolated and their structures demonstrated the presence of Lys 31–Lys 37 and Lys 7–Lys 37 cross-links in the enzymatically superactive amidinated ribonuclease.

The introduction of covalent, intramolecular cross-links into proteins of known amino acid sequence by reaction with bifunctional reagents and the subsequent identification of the location of the cross-link provide a direct chemical method for determining interresidue distances in biologically active proteins in dilute aqueous solution (Zahn and Meienhofer, 1958; Hiremath and Day, 1964; Marfey *et al.*, 1965a,b; Fasold, 1965). Although it is highly improbable that the complete three-dimensional structure of any protein can be solved by this method, the determination of distances between several pairs of residues in a given protein should greatly reduce the number of possible conformations one needs to consider in constructing three-dimensional models of that protein. The kind of information one can obtain from the characterizations of cross-linked proteins should have a very immediate significance in providing answers to the question of whether or not there are significant differences in the structure of a protein in solution as compared to its structure, determined by X-ray diffraction, in the crystalline state.

A wide variety of bifunctional protein reagents, each with its own set of advantages and disadvantages in terms of specificity, reactivity, solubility, and stability, have been studied (for a review, see Wold, 1967). The diimido esters represent a relatively new and attractive addition to the list of such reagents. Hunter

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and Ludwig (1962) showed that water-soluble imidoesters react specifically and under mild conditions with protein amino groups. The resulting amidines can be cleaved by treatment with ammonium hydroxide to yield the original amino group under conditions which will not cause hydrolysis of peptide bonds (Ludwig and Byrne, 1962). Since the pK_a of amidines is higher than that of ϵ-amino groups, an amidinated protein has the same net charge in the acid or neutral pH range as does the native protein. Wofsy and Singer (1963) have shown that exhaustive amidination of bovine serum albumin and rabbit γ-globulin with ethyl acetimidate results in little alteration of their chemical and physical properties. Dutton *et al.* (1966) have used diethyl malonimidate as a cross-linking reagent for the same two proteins and in these experiments 85% of the total free lysines could be amidinated without destroying any of the antigenic determinants in the two proteins.

In this paper we report the use of the diimido ester, dimethyl adipimidate,[1] as a cross-linking reagent for RNase. The paper describes the synthesis of the ^{14}C-labeled reagent and its lysine derivatives, the analytical methods employed in the quantitation of the reaction products, the reaction of adipimidate with RNase, and the characterization of the resulting derivative, and finally the partial elucidation of cross-linked positions in an enzymatically active monomeric derivative.

Experimental Section

Materials. RNase, type I-A (lot 95B-0330), and cytidine 2′,3′-cyclic phosphate were purchased from the Sigma Chemical Co. and used without purification. Trypsin was obtained from Pentex and 1,4-dichlorobutane from the Aldrich Chemical Co. Hexanonitrile was purchased from Eastman Kodak Co. and ϵ-*N*-carbobenzoxy-L-lysine from New England Nuclear. [^{14}C]Sodium cyanide was obtained from International Chemical and Nuclear Corp. All other reagents were of the highest available purity.

Assays. The method of Lowry *et al.* (1951) was used for protein determinations with the appropriate standard curves for either native or amidinated RNase. RNase activity toward cytidine 2′,3′-cyclic phosphate was determined by the method of Crook *et al.* (1960) and toward RNA by the method of McDonald (1955). The kinetic constants for the hydrolysis of cytidine 2′,3′-cyclic phosphate were determined in 0.1 M Tris-HCl buffer (pH 7.2) according to the method of Litt (1961). Radioactivity of all samples was assayed in a Packard Model 3000 liquid scintillation spectrometer. The sample (2 mg or less of protein, either dry or in 0.05–0.2-ml aliquots of chromatographic fractions) was placed in a counting vial and dissolved in 1 ml of Hyamine hydroxide, followed by the addition of 14 ml of a scintillation fluid composed of 4.6 g of 2,5-diphenyloxazole and 115 mg of 1,4-bis-2-(5-phenyloxazolyl)benzene in 1 l. of toluene–ethanol (4:3).

Amino Acid Analyses. Analyses were performed on acid hydrolysates prepared in 6 N HCl in evacuated (50 μ), sealed tubes at 110° for 21 hr. The hydrolysates were concentrated to dryness on a rotary evaporator and analyzed with a Spinco 120B amino acid analyzer according to the method of Spackman *et al.* (1958). Correction for destruction during hydrolysis was based on recovery values from standard amino acid mixtures hydrolyzed under identical conditions. For analyses of AA-RNase and its peptides, the short column, after emergence of arginine in the normal pH 5.28 buffer, was eluted with a pH 9.7 borate buffer, prepared by adjusting the pH of a 0.1 M boric acid–0.35 M sodium acetate solution with 5 N NaOH. This allowed the direct quantitation of AA-bislysine, which is eluted under these conditions.

Performic Acid Oxidation. Oxidation of RNase and its derivatives was performed at −7° according to the procedure described by Hirs (1956).

Tryptic Digestion. The oxidized proteins were digested with trypsin at a protein to trypsin weight ratio of 100:1 for 2 hr at 37° in 0.1 M $(NH_4)_2CO_3$ buffer (pH 8.0) as reported by Anfinsen *et al.* (1959).

Peptide mapping was performed essentially by the method outlined by Katz *et al.* (1959), with the exception that electrophoresis was conducted prior to chromatography. The salt-free sample (approximately 1 mg of peptide material in 0.01 ml of buffer) was applied to a 350 × 400 mm sheet of Whatman 3MM chromatography paper at the center of a line drawn parallel to and 3.5 in. from the 400-mm edge. The applied sample was subjected to electrophoresis, along the line of its application, at 2000 v for 1 hr in pyridine–acetic acid buffer (pH 6.5) (pyridine–glacial acetic acid–H_2O, 5:0.2:95). A Brinkmann Pherograph high-voltage electrophoretic apparatus was employed. After air drying the paper for 4 hr and perforating the edge to allow even solvent flow from the chromatogram, it was developed by the descending method for 12 hr using the top layer obtained on partitioning 1-butanol–H_2O–glacial acetic acid (4:5:1). The peptides were visualized by dipping the dried chromatograms in 0.3% ethanolic ninhydrin solution and heating at 80°.

For the determination of amino acid composition of peptides from peptide maps, the papers were thoroughly dried at 70°, dipped in a 0.05% ethanolic ninhydrin solution, and heated at 60° until the components were faintly visible. The ninhydrin-positive spots were cut out, washed with acetone to remove excess ninhydrin, eluted with 6 N HCl, hydrolyzed, and analyzed.

Autoradiography. Autoradiograms of peptide maps from AA-RNase were prepared using Kodak No-Screen Medical X-Ray film with 1-week exposure time.

[1] Abbreviations used: RNase, bovine pancreatic ribonuclease A; DMA, dimethyl adipimidate; AA-RNase, adipamidino-RNase (the reaction product of DMA and RNase); AA-bislysine, N_ϵ,N_ϵ-adipamidinobis-L-lysine; MH, methyl hexanoimidate; HA-RNase, hexanoamidino-RNase (the reaction product of MH and RNase.

[^{14}C]*Dimethyl Adipimidate.* A mixture of [^{14}C]NaCN (1 mc in 0.5 g, 10.2 mmoles), which had been dried for 12 hr at 110°, and 5 ml of dimethyl sulfoxide was brought to 90°, and 0.54 g (4.25 mmoles) of 1,4-dichlorobutane was added, with vigorous stirring. An immediate exothermic reaction ensued as indicated by a rise in temperature to 130°. After the reaction mixture had cooled to 40°, 20 ml of $CHCl_3$ was added, and the solution was washed with 30 ml of saturated NaCl. The $CHCl_3$ layer was collected, and the aqueous phase was extracted with two 20-ml portions of $CHCl_3$. The combined $CHCl_3$ solutions were dried over Na_2SO_4 and concentrated to yield 0.34 g (74%) of adiponitrile. An ice-cold solution of the adiponitrile in a mixture of 8 ml of dioxane and 1 ml of methanol both of which had been dried over CaH_2, was saturated with dry HCl gas during 15 min. The reaction mixture was left in an ice bath for 12 hr, at which time the diimido ester dihydrochloride was precipitated by the addition of ether (15 ml), collected by filtration, and washed exhaustively on the filter pad with methanol–ether (1:3), care being taken not to expose the material to the atmosphere until the excess HCl had been completely removed. Recrystallization from methanol–ether gave 625 mg (83%) of [^{14}C]DMA dihydrochloride, a sample of which consumed 2 equiv of NaOH during titration. During melting point determinations it was converted to adipamide, mp 218–220°, a phenomenon consistent with previously described properties of imido esters (Roger and Neilson, 1961).

Anal. Calcd for $C_8H_{18}Cl_2N_2O_2$ (245.16): C, 39.19; H, 7.41; N, 11.42. Found: C, 39.42; H, 7.51; N, 11.38.

These procedures for preparing adiponitrile and DMA are similar to those described by Smiley and Arnold (1960) and McElvain and Schroeder (1949), respectively.

Methyl Hexanoimidate. A solution containing 5 g of hexanenitrile in a mixture of 5 ml of methanol and 50 ml of ether was saturated with dry HCl gas at 0°, and after 8 hr the reaction mixture was concentrated to dryness at 30°. The addition of ether to the residue gave 7.29 (84%) of a crystalline product, mp 100–102° dec.

Anal. Calcd for $C_7H_{15}ClNO$ (165.67): C, 50.70; H, 9.74; N, 8.45. Found: C, 50.82; H, 9.84; N, 8.51.

Synthesis and Characterization of N_ϵ,N_ϵ-Adipamidino-bis-L-lysine. α-*N*-Acetyl-L-lysine was prepared from ϵ-*N*-carbobenzoxy-L-lysine by the method of Neuberger and Sanger (1943). To a solution of 2 g (10.6 mmoles) of α-*N*-acetyl-L-lysine in a mixture of 3 ml of H_2O, 30 ml of methanol, and 3 ml of triethylamine was added with continuous stirring at room temperature 1.5 g (6 mmoles) of DMA dihydrochloride in 100-mg portions during a 30-min period. Two hours after the addition of reagent had been completed, 50 ml of H_2O was added to the reaction mixture, and the resulting solution was extracted with two 50-ml portions of $CHCl_3$. The water layer was concentrated to dryness, and the residue was heated in 50 ml of 6 N HCl at 90° for 8 hr, at which time the solution was taken to dryness. The resulting syrup was dissolved in 5 ml of H_2O, and the solution was neutralized with 5 N NaOH. Paper chromatography in 1-butanol–glacial acetic acid–H_2O (7:2:5) revealed three ninhydrin-positive components (R_F 0.06, 0.12, and 0.55) in addition to lysine (R_F 0.25). The above, neutralized solution was applied to a 1.5 × 40 cm column of Bio-Rad AG50W-X4 (Na^+) resin. The column was eluted with a linear gradient of 0–1.5 M NaCl in 0.35 M $NaHCO_3$ (pH 7.5) (750 ml in both mixing chamber and reservoir). The flow rate was 45 ml/hr, and 15-ml fractions were collected. Aliquots (0.05 ml) of each fraction were assayed by the ninhydrin procedure of Moore and Stein (1954). The results are given in Figure 1. Paper chromatography in the previously used solvent identified B as lysine and A, C, and D as the components having R_F of 0.55, 0.06, and 0.12, respectively. The fractions containing D, subsequently identified as the desired AA-bislysine derivative, were pooled and, after adjusting the pH to 3.0 with 6 N HCl, concentrated to dryness. The organic material was extracted with methanol from the salts, and the methanol extracts were concentrated to dryness. The AA-bislysine was freed of remaining salts by passage through a 2.5 × 100 cm Sephadex G-10 column equilibrated with water. Concentration of the ninhydrin-positive fractions yielded 1.6 g (64%) of a thick, colorless syrup. Attempts to crystallize the product were unsuccessful, and its identity and purity were, therefore, established by chromatography, degradation, and derivatization.

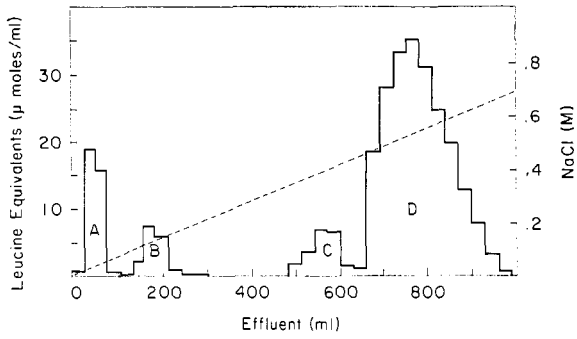


FIGURE 1: Ion-exchange purification of AA-bislysine. The experimental details are given in the text.

A 0.4-μmole (based on weight) sample of AA-bislysine was subjected to chromatography on the short column of the amino acid analyzer. The only ninhydrin-positive material eluted by the normal pH 5.28 buffer was a trace of ammonia. The dilysine derivative was eluted as a single, symmetrical peak (Figure 2A) which appeared 40 min after changing to the borate buffer (see Amino Acid Analyses section). A duplicate sample, on treatment with concentrated ammonium hydroxide at room temperature for 12 hr, gave rise to 0.76 μmole (95%) of lysine as measured on the amino acid analyzer (Figure 2B). No other components, except ammonia, were detected. The observed ninhydrin color yield of AA-bislysine was equivalent to that of lysine.

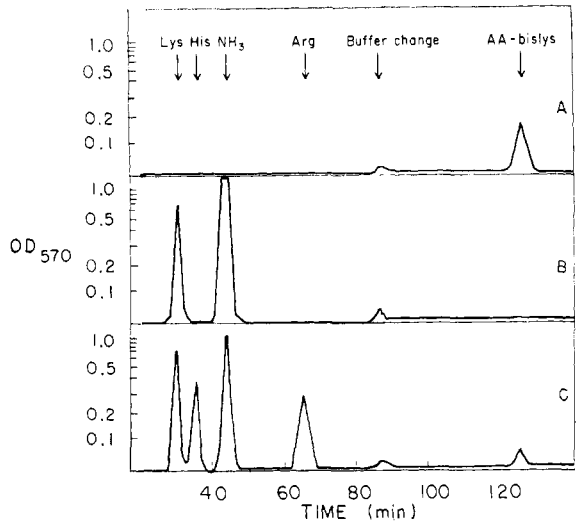


FIGURE 2: Ion-exchange chromatography of AA-bislysine on the Spinco amino acid analyzer short column, changing to the 0.1 M boric acid–0.35 M sodium acetate buffer (pH 9.7) after the completion of the normal short-column run. (A) Chromatography of purified AA-bislysine; (B) chromatography of AA-bislysine after treatment with concentrated ammonium hydroxide at room temperature for 12 hr; (C) a typical analysis of the acid hydrolysate of AA-RNase monomer.

Dinitrophenylation followed by ammonium hydroxide treatment of AA-bislysine resulted in the formation of α-DNP-lysine as the only component detectable by paper chromatography using the organic phase from 1-butanol–*n*-butyl acetate–1% ammonium hydroxide (1:2:3) as the solvent (Mellon *et al.*, 1953). It is felt that these experiments unequivocally establish the identity of component D in Figure 1 as AA-bislysine. Components A and C have not been characterized.

Recovery values for AA-bislysine after acid hydrolysis were determined by heating the proper quantities of the synthetic AA-bislysine at 110° in 6 N HCl for 21 hr. Calculations were based on the amount of AA-bislysine present before and after hydrolysis, as measured on the amino acid analyzer. At concentrations of 3.4×10^{-4} and 6×10^{-5} M (equivalent to the concentration range of AA-bislysine present in the AA-RNase samples hydrolyzed), the percentage of recovery was 86 and 83%, respectively. The destruction was independent of the presence of air during hydrolysis.

Molecular Weight Determinations. Equilibrium ultracentrifugation, performed by the method of Richards and Schachman (1959) using the Spinco Model E ultracentrifuge equipped with an interference optical system, was employed to determine the molecular weights of native and amidinated RNase. The centrifugations were conducted for 24 hr at 20° and 18,000 rpm. The protein concentration was 2 mg/ml in 0.1 M KCl buffered with 0.01 M potassium phosphate (pH 6.8).

Amidination of RNase. Solid [^{14}C]DMA dihydrochloride (27 mg, 110 μmoles) was added in 2-mg portions at 5-min intervals to a continuously stirred solution of 500 mg (36.7 μmoles) of RNase in 50 ml of 0.1 M sodium phosphate (pH 10.5) at room temperature. Sodium hydroxide (0.1 N) was added as required to maintain the pH at 10.5. One hour after the addition of diimido ester had been completed, the protein solution was desalted on a 1 × 60 in. column of Sephadex G-25 equilibrated with 0.01 M ammonium acetate. The RNase-containing fractions were pooled and lyophilized giving 485 mg of product. A 100-mg sample of RNase was amidinated with 6 molar equiv of MH, the monofunctional methyl hexanoimidate, by the same procedure.

Fractionation of Amidinated RNase. A solution of AA-RNase (200 mg in 15 ml of 0.01 M ammonium acetate) and a solution of HA-RNase (100 mg in 7 ml of 0.01 M ammonium acetate) were subjected to gel filtration on a 1 × 72 in. column of Sephadex G-75 in 0.01 N ammonium acetate at a flow rate of 23 ml/hr. Fractions of 11.5 ml were collected. The positions at which monomers, dimers, and higher aggregates of RNase emerged were determined by passing through the same column a solution of RNase which had been aggregated by lyophilization from 50% acetic acid as described by Crestfield *et al.* (1962).

Characterization of Amidinated RNase Fractions. Each isolated fraction of AA-RNase was characterized with respect to the extent of the amidination reaction. The number of moles of reagent incorporated and the number of cross-links formed were determined directly from the radioactive count of each sample and from the amount of AA-bislysine present in acid hydrolysates, respectively. The total number of moles of lysine residues reacted with the reagent was obtained in the following manner. Amidinated protein (10 mg) and $NaHCO_3$ (10 mg) were dissolved in 0.2 ml of water and 0.4 ml of a solution of 5% (v/v) of fluorodinitrobenzene in ethanol was added. The reaction mixture was shaken vigorously at room temperature for 2 hr, then acidified with concentrated HCl, diluted with 3 ml of water, and extracted with ether. The protein precipitate in the aqueous phase was collected by centrifugation and washed with water, ethanol, and ether. After acid hydrolysis, the hydrolysate was taken to dryness in the usual manner, and then treated with 1 ml of concentrated ammonium hydroxide–glacial acetic acid (15:2) at room temperature overnight. This latter treatment leads to complete cleavage of and liberation of free lysine from the amidine derivatives (Ludwig and Byrne, 1962), and amino acid analysis of these samples thus gave a quantitative estimate of the lysine residues which were inaccessibly to dinitrophenylation and were liberated after deamidination. Control samples of unreacted RNase treated in the same manner gave 0.35 mole of free lysine/mole of enzyme, and the AA-RNase lysine values given in Table I as "lysine modified" have been corrected for this background value.

Isolation and Characterization of Cross-Linked Peptides from Tryptic Digests of Oxidized AA-RNase. The desired peptides were purified by preparative

TABLE 1: Analytical Data for Amidinated RNase Derivatives.

Reagent (molar ratio of reagent:protein)	Fraction (% of total)	Reagent Incorp[a] (moles/mole of protein)	Lysine Modified[b] (moles/mole of protein)	AA-bislysine[c] (moles/mole of protein)	Enzymatic Act. (% of native)
DMA (3:1)	Monomer (47)	2.3	3.2	0.88	160[d], 85[e]
	Dimer (29)	2.7	4.0	1.05	135
	Aggregate (24)	2.8	4.1	1.05	130
MH (6:1)	Monomer (100)		4.0		100

[a] Based on radioactivity incorporated. [b] Based on free lysine in the amidinated derivatives after exhaustive dinitrophenylation, acid hydrolysis, and deamidination with NH_4OH. [c] Determined by direct analysis of acid hydrolysates on the amino acid analyzer (see Figure 2C) and corrected for destruction during hydrolysis. [d] Activity toward cytidine 2',3'-cyclic phosphate. [e] Activity toward RNA.

electrophoresis and chromatography employing conditions identical with those used for peptide mapping. A solution of tryptic peptides from 15 mg of performic acid oxidized, AA-RNase monomer in 0.2 ml of pyridine–acetic acid buffer (pH 6.5) was applied as a narrow band 20 cm long at the center of the chromatography paper and subjected to electrophoresis. After air drying the paper, radioactive bands were detected by scanning (with a Packard radiochromatogram scanner) 1-cm wide strips cut from each end of and perpendicular to the applied band. The labeled peptides were eluted from the paper with H_2O and the eluents were lyophilized. The dry residues were taken up in 0.2 ml of pyridine–acetic acid buffer (pH 6.5), applied as narrow bands to chromatography paper, and chromatographed. The radioactive bands were detected and eluted as described above. After lyophilization, a portion (25%) of each purified peptide was hydrolyzed for the determination of amino acid composition. The remainder was treated with 0.2 ml of concentrated ammonium hydroxide–glacial acetic acid (15:2) at room temperature for 8 hr (Ludwig and Byrne, 1962), at which time 10 ml of H_2O was added and the solutions were lyophilized. The remaining ammonium acetate was removed by three additional lyophilizations from 10 ml of H_2O. The peptides were then dissolved in 0.4 ml of 0.1 M ammonium carbonate and digested with 0.02 mg of trypsin. These digests were subjected to peptide mapping and the component peptides were identified by their positions on the map.

Results

The gel filtration elution patterns of the desalted and lyophilized AA-RNase and HA-RNase reaction products on Sephadex G-75 are shown in Figure 3. In the same figure is also shown a reference elution pattern of native RNase, RNase dimer, and more highly aggregated species obtained by lyophilization of RNase A from 50% acetic acid (Crestfield *et al.*, 1962). It is evident that the AA-RNase product contains both aggregated (peak A), dimeric (peak B), and monomeric (peak C) reaction products. On lyophilization, peaks A, B, and C yielded 45, 55, and 87 mg, respectively. The fact that HA-RNase emerged as a single peak coincident with the monomer peak (Figure 3) suggests that the dimeric and the aggregated AA-RNase products are formed through intermolecular cross-linking with the bifunctional reagent. It should be emphasized that a control sample of AA-RNase reaction mixture subjected to gel filtration on the same column prior to desalting and lyophilization gave an identical elution pattern, thus demonstrating that the aggregated species were not a result of lyophilization. Fractions A–C (Figure 3) were characterized with respect to quantity of reagent incorporated, number of cross-links introduced, number of lysine residues

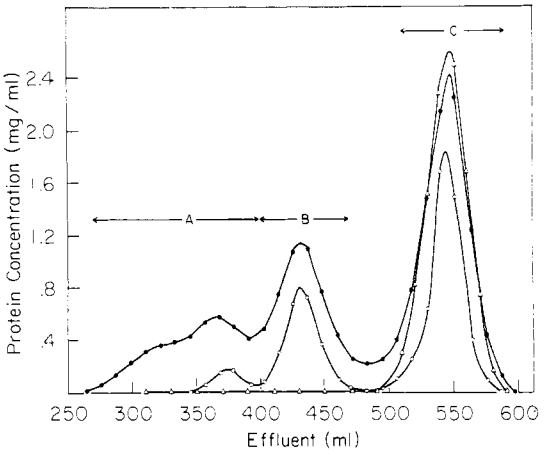


FIGURE 3: Gel filtration of RNase derivatives on Sephadex G-75. (O—O) Aggregated RNase; (●—●) AA-RNase (200 mg); (△-△) HA-RNase (100 mg). The experimental details are given in the text. A–C refer to the pooled fractions of AA-RNase only.

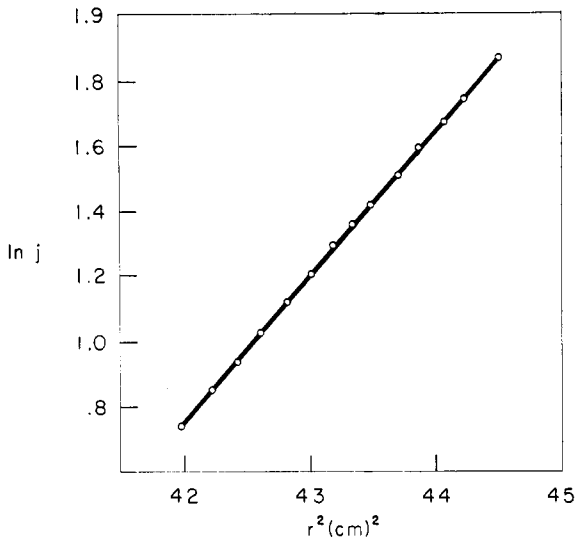


FIGURE 4: Test for homogeneity of AA-RNase monomer with respect to weight-average molecular weight. Experimental details given in text.

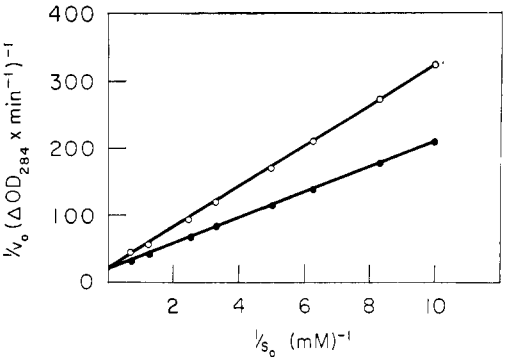


FIGURE 6: Lineweaver–Burk plots for the hydrolysis of cytidine 2′,3′-cyclic phosphate catalyzed by native RNase (O—O) and AA-RNase (●—●). Enzyme concentration, 3.0×10^{-7} M. The experimental details are given in the text. The K_m values obtained from the plot are: K_m (RNase) = 1.7×10^{-3} M and K_m(AA-RNase) = 1.1×10^{-3} M.

modified, and enzymic activity, and the results are given in Table I.

In all this work, the direct analysis of AA-bislysine on the amino acid analyzer was invaluable (Figure 2). Although the AA-bislysine peak (from protein hydrolysates) observed with the Spinco Model 120B amino acid analyzer was symmetrical (Figure 2C), during recent analyses of the same samples on the Model 120C analyzer a shoulder representing approximately one-third of the total area was seen on the descending side of the peak. Since partial resolution of the standard AA-bislysine was never observed, it is assumed that the lesser component is not an artifact and that it represents a N_α,N_ϵ-dilysine component arising from a cross-link involving the α-amino group of the N-terminal lysyl residue. This compound would probably be eluted after AA-bislysine due to the higher pK of the ϵ-amino group as compared to the α-amino group. It should be emphasized that with the exception of lysine the amino acid content of all the derivatives was identical to that of native RNase. This, together with the internally consistent analytical data in Table I, demonstrates the specificity of the reaction.

All the subsequent work was carried out with the monomeric fraction of AA-RNase. The weight-average molecular weight of this fraction, as determined by sedimentation equilibrium analysis in the ultracentrifuge was 14,000 ± 500 (native RNase, in a parallel run gave 13,200 ± 500), and the data in Figure 4 demonstrate the molecular weight homogeneity of the sample. Electrophoresis on cellulose acetate strips gave no evidence for the presence of unreacted, native RNase in the AA-RNase monomer fraction, but the electrophoretic patterns indicate that the derivative was heterogeneous (Figure 5). Partial resolution of different

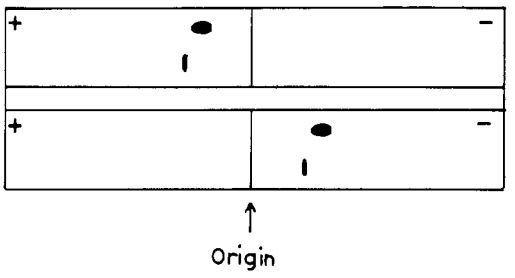


FIGURE 5: Electrophoresis of AA-RNase monomer (top of each strip) and native RNase on cellulose acetate strips. Conditions for upper strip: 0.05 M sodium carbonate buffer (pH 10.5) 1 hr at 300 v. Conditions for bottom strip: 0.02 M sodium phosphate buffer (pH 6.5) 3 hr at 300 v.

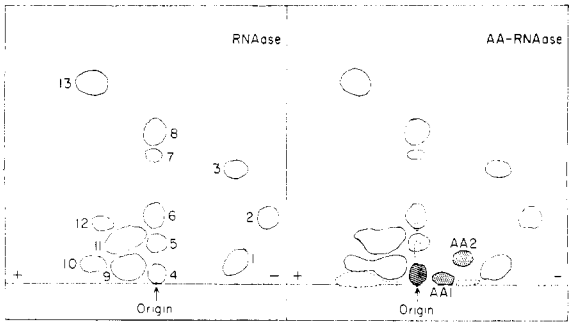


FIGURE 7: Peptide maps of native RNase and AA-RNase monomer. Ninhydrin-positive peptides are enclosed with solid lines. Radioactive peptides (detected by autoradiography) are cross-hatched. Faint radioactive components are enclosed with broken lines. The experimental details are given in the text.

components in this AA-RNase fraction has been achieved on sulfoethyl-Sephadex, but no method has yet been found which is suitable for the isolation of homogeneous derivatives in quantities sufficient for degradative studies.

The above data demonstrate that the isolated fraction of AA-RNase is indeed a monomeric derivative of ribonuclease, containing 1 mole of bifunctionally reacted adipamidine cross-link and 1.3–1.4 moles of monofunctionally attached adipamidine/mole of enzyme. Only lysyl residues were involved in the reaction, but from the electrophoretic heterogeneity it appears that the fraction is a mixture of derivatives, differing, most likely, both in degree of substitution and in the specific lysyl residues involved in the reaction. Since the monofunctional analog of DMA when reacted with RNase under the same conditions did not give an alteration in the activity of the derivative, we tentatively conclude that the increased activity of AA-RNase toward cytidine 2,3′-cyclic phosphate is a specific result of the AA cross-link.

A kinetic analysis of AA-RNase in comparison with native RNase is given in Figure 6, and the data show that only the K_m was affected by the introduction of the reagent.

Peptide maps of performic acid oxidized, trypsin-digested native RNase, and AA-RNase monomer are shown in Figure 7. The components on the map of native RNase were eluted and identified by quantitative amino acid analyses, so that purified cross-linked peptides could be unequivocally identified by peptide mapping after removal of the cross-link and digestion with trypsin. The results of the analyses are given in Table II.

TABLE II: Identification of Peptides from Peptide Map of Performic Acid Oxidized, Trypsin-Digested RNase.

Peptide (from Fig 7)	Residue Composition[a]	Nomenclature of Hirs *et al.* (1956)
1	1–7 + 99–104	*O*-Trp 10 + *O*-Trp 8
2	32–33	*O*-Trp 12
3	34–37	*O*-Trp 11
4	11–31	*O*-Trp 4
5	86–91 + 86–98	*O*-Trp 6 +
6	62–66 + 38–39	*O*-Trp 5 + *O*-Trp 7
7	92–98	*O*-Trp 14
8	8–10	*O*-Trp 15
9	*b*	
10	67–85	*O*-Trp 2
11	40–61	*O*-Trp 9
12	*c*	
13	105–124	*O*-Trp 16

[a] For complete amino acid sequence (see Smyth *et al.*, 1963). [b] Analysis similar to that of peptide 11. [c] Analysis similar to that of peptide 10.

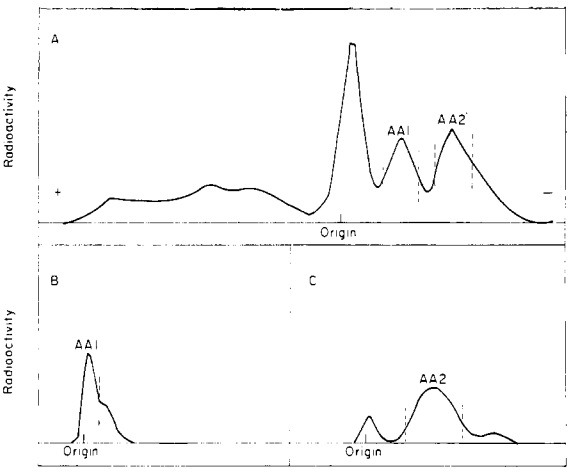


FIGURE 8: (A) Distribution of radioactivity after preparative electrophoresis of tryptic digests of AA-RNase monomer. (B) Distribution of radioactivity after paper chromatography of fraction AA1 from A. (C) Distribution of radioactivity after paper chromatography of fraction AA2 from A. The experimental details are given in the text.

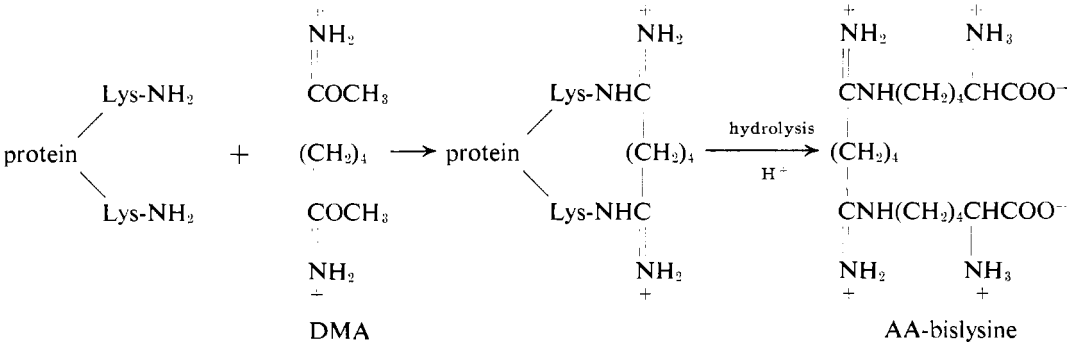
Since amino acid analyses of the radioactive peptides eluted from the peptide map were equivocal due to the small quantities present, quantities of peptides AA1 and AA2 (Figure 7) sufficient for characterization were isolated by preparative high-voltage electrophoresis followed by preparative paper chromatography. The distribution of radioactivity after electrophoresis and after chromatography was determined by scanning and is illustrated in Figure 8. One-fourth of the purified samples of components AA1 and AA2 in Figure 8 were hydrolyzed and subjected to amino acid analysis, the results of which are recorded in Table III.

The amino acid content of peptide AA1 is consistent with that of a peptide comprising residues 11–39 and containing 1 mole of AA-bislysine. Such a peptide would be expected from a RNase derivative containing a Lys 31–Lys 37 cross-link.

The amino acid analysis of an equimolar mixture of two peptides, one of which contained residues 1–10 and 34–39 and the other residues 1–7, would be in agreement with that found for component AA2. A Lys 7–Lys 37 cross-link would account for the presence of a peptide-containing residues 1–10 and 34–39. Monofunctional modification of the N-terminal lysine would give rise to a peptide composed of residues 1–7.

The remaining three-fourths of the purified peptides was treated with ammonium hydroxide to remove the cross-links and then digested with trypsin. Peptide mapping demonstrated the release of peptides *O*-Trp 4, 7, 11, and 12 from component AA1, and peptides *O*-Trp 7, 10, 11, and 12 from component AA2. These results confirm the above identifications based on amino acid analyses.

SCHEME I



Discussion

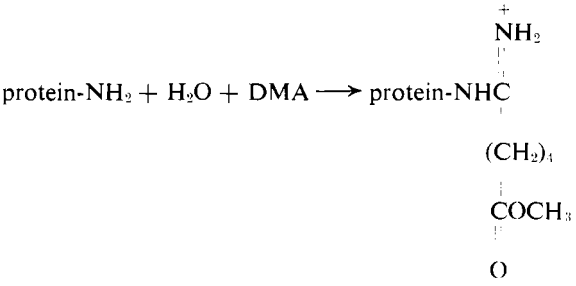
The work of Hunter and Ludwig (1962) on the reaction of imido esters with amino acids and proteins demonstrated the specificity of this type of reagent and suggested that bisamidines would be the most likely product of the reaction between DMA and RNase. Direct evidence for the formation of cross-links in the protein by this reaction should be provided by the identification of AA-bislysine in acid hydrolysates of the protein derivative (Scheme I). Although the cross-link is illustrated as being intramolecular, the formation of intermolecular bridges must also be considered.

The fact that imido esters hydrolyze under mild conditions (Roger and Neilson, 1961) could give rise to monofunctionally amidinated RNase species in which one imido ester group of the DMA molecule reacted with water and the other with the protein.

TABLE III: Amino Acid Analyses of Peptides AA1 and AA2.

	No. of Residues			
	Peptide 1		Peptide 2	
Amino Acid	Found[a]	Calcd[b]	Found[a]	Calcd[c]
AA-bislysine	0.7	1	0.6	1
Lys	0		2.1	2
Arg	1.6	2	2.0	2
Asp	4.6	5	2.1	2
Thr	2.3	2	3.2	3
Glu	2.3	2	3.5	3
Ala	2.3	2	6.0	6
Leu	0.95	1	1.0	1
Phe	0.0		1.1	1
Tyr	1.0	1	0.0	
His	0.9	1	0.0	
Cysteic	0.8	1	0.0	
Ser	6.3	7	0.0	
Met-SO_2	2.5	3	0.0	

[a] The following corrections have been made: (1) destruction of AA-bisLys, Thr, and Ser during hydrolysis; (2) lysine due to hydrolysis of AA-bisLys; (3) Gly and Ala found in chromatography paper. Gly, Pro, Val, and Ile were not found in significant amounts in either peptide. [b] Peptide corresponding to residues 11–39 (31–37 bridge). [c] Equimolar amounts of two peptides corresponding to residues 1–10, 34–39 (7–37 bridge), and 1–7 (monofunctional amidination of Lys 1), respectively.



The analytical methods used in this work, making possible the accurate determination of the number of moles of reagent incorporated (from radioactivity measurements), the number of amino acid residues reacted (from the difference in amino acid content before and after reaction), and the quantity of AA-bislysine formed in the reaction (by direct analysis, corrected for destruction during acid hydrolysis) permit a very precise characterization of the reaction product in terms of the relative extent of monofunctional and bifunctional substitution as well as the total extent of the reaction. Thus, the presence of AA-bislysine in acid hydrolysates of AA-RNase monomer clearly establishes that intramolecular cross-links have been introduced into the enzyme. However, the ratio of lysyl residues modified to moles of reagent incorporated is 1.39 (Table I) indicating that only 39% of the incorporated DMA is present as AA-bislysine. The number of introduced cross-links as calculated from direct measurement of AA-bislysine by automatic amino acid analysis indicates 37% of the incorporated DMA has reacted bifunctionally. Thus, the degree of cross-linking based

on both lysyl residues modified in conjunction with reagent incorporated and on AA-bislysine content are in good agreement.

Any acid-labile derivative formed in the reaction might not be detected by the analytical methods used. It is in fact possible that the presumed monofunctional substitution could be a result of a Lys–AA-X cross-link, in which the AA-X bond is readily hydrolyzed. A careful analysis of all the radioactive components in the tryptic peptide maps should rule out this possibility, as any peptide resulting from a Lys–AA-X cross-link presumably can be recognized and accounted for on the maps. Based on our present analyses, it can be concluded that Lys–AA-X cross-links are at the most very minor components in the derivative, and we feel that our data confirm the reported specificity of imido esters (Hunter and Ludwig, 1962).

The formation of dimers and higher molecular weight aggregates of RNase during the reaction with DMA is probably due to the formation of intermolecular cross-links, as these aggregates cannot be dissociated under conditions which induce the dissociation of noncovalent aggregates (Crestfield *et al.*, 1962). The fact that no aggregates were formed during the reaction of RNase with the monofunctional imido ester (MH) is also strong (although perhaps indirect) evidence that covalent intermolecular cross-links led to the aggregates. When the RNase concentration in the reaction mixture was lowered to 1 mg/ml in an attempt to decrease the extensive intermolecular cross-linking, the resulting monomeric fraction of AA-RNase contained less than 0.2 mole of AA-bislysine/mole of enzyme. However, more optimal reaction conditions for cross-linked monomer formation can undoubtedly be developed. The dimers and high molecular weight products have not yet been characterized. It would appear, however, that they represent very interesting models for the study of protein–protein interaction, and warrant further attention.

If the introduction of intramolecular cross-links into proteins is to be used successfully as a method for determining interresidue distances, it is essential that the chemical modification does not induce major conformational changes. Since maintenance of a protein's biological activity is an indication that gross conformation changes have not occurred, we hoped to obtain RNase derivatives with enzymic properties similar to those of the native enzyme. For this reason the modification of RNase with DMA has been conducted in the presence of phosphate ions, which reportedly (Cooke *et al.*, 1963) protect lysine 41, a residue essential to the maintenance of structural integrity and enzymic activity (Hirs, 1962; Marfey *et al.*, 1965a,b). A pH of 10.5 was chosen on the basis of the reported pH optimum (10–10.5) for the reaction of methyl acetimidate, an imido ester whose reactivity with ϵ-amino groups should be similar to that of DMA (Hunter and Ludwig, 1962).

The increase in specific activity toward cytidine 2′,3′-cyclic phosphate which accompanied the amidination of RNase is due to a decreased K_m (Figure 7). In an attempt to ascertain whether this decrease was due merely to the conversion of an amino group near the active site to the more basic amidine, or due to an alteration or fixation of conformation imposed by the introduced cross-links, the cross-linked AA-RNase was again compared to the monofunctionally substituted HA-RNase derivative. The fact that the specific activity of HA-RNase was identical with that of native RNase suggests that the observed decrease in K_m of AA-RNase is a direct result of cross-linking. Of course, it can be argued that the same lysyl residues of RNase were not modified with MH as with DMA. It is even possible that the ester functions present in AA-RNase, resulting from hydrolysis of one end of the DMA molecule, induced the change in specific activity. However, the first interpretation, that the superactivity is a direct result of a cross-link, is by far the most attractive, and it would appear, also the most reasonable. The notion that molecular rigidity introduced into the protein *via* the covalent cross-link could result in a higher affinity for one substrate (cytidine 2,3-cyclic phosphate), and a reduction in the activity toward the other (RNA) has some very interesting implications related to induced fit enzyme models. It may also be taken in support for the idea that RNase has separate sites (or different conformation forms) for the two substrates, and it is hoped that the use of bifunctional reagents under different reaction conditions may give unequivocal evidence on these points.

Two cross-linked peptides were obtained by preparative paper electrophoresis and chromatography of trypsin digests of the unresolved AA-RNase monomeric species after performic acid oxidation. Amino acid analyses (Table III) demonstrated that one of these contained residues 11–39. A bridge between lysine 31 and lysine 37 in the RNase molecule would result in the release of a peptide having the determined composition because the peptide bond involving the carboxyl group of the amidinated lysyl residue is resistant to trypsin-catalyzed hydrolysis (Ludwig and Byrne, 1962). Since the maximum distance that the reagent can span is 8.6 A and the minimum distance between the ϵ-amino groups of lysyl residues 31 and 37 in a fully extended peptide chain is 12 A, this region of the RNase molecule must exist in a folded state.

The other cross-linked peptide contained residues 1–10 and 34–39, demonstrating the presence of a bridge between lysines 7 and 37 in AA-RNase. This peptide was obtained as a mixture with a second peptide composed of residues 1–7, which presumably arose through monofunctional modification of the N-terminal lysyl residue. As the close proximity of lysyl residues 7 and 41 in the RNase molecule has been inferred from the enhancement of reactivity of the ϵ-amino group at position 7 which accompanies the arylation with 1-fluoro-2,4-dinitrobenzene of the ϵ-amino group at position 41 (Hirs *et al.*, 1956; Hirs, 1962) and established unequivocally by the formation of a 7–41 cross-link upon reaction with 1,5-difluoro-2,4-dinitrobenzene (Marfey *et al.*, 1965b), the finding in this investigation of a 7–37 cross-linking is not surprising.

It should be emphasized that the assigned structures of the cross-linked peptides were confirmed by removal of the cross-link followed by trypsin digestion and peptide mapping, giving in both cases only the expected tryptic peptides. Hammes and Scheraga (1966) have pointed out that the positions of these cross-links are consistent with their proposed model which is based on previously reported side-chain interactions. Of even greater significance is the fact that the two cross-links found in this work appear to be in complete accord with the interresidue distances estimated from the published results of X-ray diffraction studies on both RNase A (Kartha *et al.*, 1967) and RNase S (Wyckoff *et al.*, 1967) crystals.

From the pattern obtained on scanning the paper electrophoretogram of the AA-RNase trypsin digest for radioactivity (Figure 8) and from the quantity of AA-bislysine recovered after hydrolysis of each sample, it was estimated that 32% of the AA-RNase monomers contained a 31–37 cross-link and 20% a 7–37 cross-link. If the increased specific activity of AA-RNase is due to the introduction of a single cross-link into only one of these two positions, then that derivative actually has a specific activity 2.5–3.5 times greater than that of the native enzyme. The separation of the various AA-RNase derivatives in the original reaction mixture must be accomplished before any firm conclusion on this point can be reached.

This investigation clearly demonstrated that diimido esters can be utilized as cross-linking protein reagents. The ease of preparing a wide variety of ^{14}C-labeled diimido esters differing in their chemical reactivity and capable of spanning various distances may prove to be the greatest single advantage of this group of bifunctional reagents.

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