Modification of the Carboxyl Groups of Ribonuclease by Attachment of Glycine or Alanylglycine*

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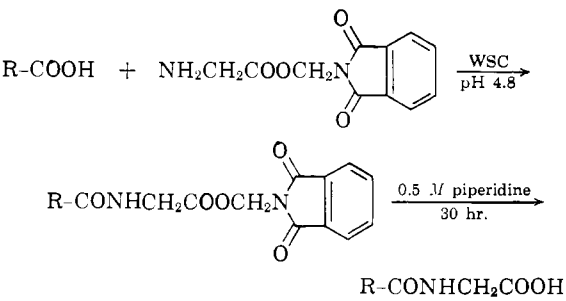
ABSTRACT: Bovine pancreatic ribonuclease (RNase) was modified by binding glycine or alanylglycine to the carboxyl functions of the enzyme, making use of the phthalimidomethyl group for the reversible blocking of the glycine or dipeptide carboxyl groups and of a water-soluble carbodiimide for the coupling reaction. In the resulting RNase-glycine derivative all the eleven carboxyl groups of RNase reacted with glycine, while in the RNase-AlaGly derivative only eight carboxyl groups reacted. In both cases the electrical charges due to the carboxylate ions have not been abolished, even though their steric positions were somewhat displaced. RNase-glycine had an ultraviolet absorption spectrum differing from that of native RNase, and on spectrophotometric titration all six tyrosine residues were available for ionization. This derivative was inactive on RNA but possessed almost all the activity of the native enzyme on cytidine 2',3'-cyclic phosphate. The activity was abolished upon full reduction, but was completely recovered after reoxidation of the derivative. The preparation modified by the attachment of the alanylglycine peptide was totally inactive.

The chemical modification of various reactive groups in proteins has been used extensively in studies on the correlation between their structure and biological activity. This approach has been applied only to a limited extent for the estimation of the role of carboxyl groups, with the exception of the progressive degradation by carboxypeptidase which involved the terminal α-carboxyl groups exclusively (Potts *et al.*, 1964; Harris, 1952; Westhead and Boyer, 1963; Wu and Schultz, 1965).

Among the early methods used for the blocking of the carboxyl groups of proteins, esterification with methanol in hydrochloric acid (Fraenkel-Conrat and Olcott, 1945) and with diazomethane (Herriot, 1947) should be mentioned. The conversion of all (Sela *et al.*, 1957) or most (Broomfield *et al.*, 1965) of the carboxyl groups of bovine pancreatic RNase into methyl esters resulted in the loss of activity on RNA. A limited esterification of RNase has been accomplished recently with diazoacetoglycinamide (Riehm and Scheraga, 1965). Sheehan and Hlavka (1956) have first suggested the use of water-soluble carbodiimide for the selective modification of the carboxyl groups of proteins. Riehm and Scheraga (1966) have treated RNase with 1-cyclohexyl-3-(2-morpholinethyl)carbodiimide metho-*p*-toluenesulfonate and isolated from the reaction product five chromatographically distinguishable components. Each of these components possessed enzymic activity, although lower than that of the native enzyme. Most recently Hoare and Koshland (1966) have reported a procedure for rapid and quantitative modification of the carboxyl groups of proteins (such as trypsin, chymotrypsin, and lysozyme) under mild conditions by making use of *N*-benzyl-*N'*-3-(dimethylaminopropyl)carbodiimide as the activating carbodiimide and glycine methyl ester as the modifying reagent.

In an independent study, to be reported here, we have modified selectively the carboxyl groups of RNase, either with glycine *N*-phthalimidomethyl ester or with L-alanylglycine *N*-phthalimidomethyl ester, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as the activating carbodiimide. The choice of the *N*-phthalimidomethyl group for the protection of the glycine (or peptide) carboxyl groups permitted its removal from the modified RNase under mild conditions (Wilchek *et al.*, 1966), yielding RNase derivatives in which either glycine or alanylglycine is attached to the carboxyl groups of the protein. The reaction, using glycine as an example, is given in the following scheme.



A derivative containing 11 glycine residues attached per

* From The Weizmann Institute of Science, Rehovoth, Israel. *Received September 26, 1966.* This investigation was supported in part by Agreement 415100 and Grant AM 5098 from the National Institutes of Health, U. S. Public Health Service.

RNase molecule (to be denoted RNase-glycine[1,2]) was completely inactive toward RNA, but possessed 93% of the activity of unmodified RNase on cytidine 2',3'-cyclic phosphate. No "buried" tyrosines could be detected spectrophotometrically in this derivative. The attachment of eight alanylglycines per average RNase molecule resulted in the total loss of activity toward either substrate.

Experimental Section

Materials

Bovine pancreatic RNase, type I-A, five times recrystallized, yeast RNA, type XI, and cytidine 2',3'-cyclic phosphate were obtained from Sigma, St. Louis, and used without further purification. The water-soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC[2]), was a product of Ott Chemicals, Muskegon, Mich. Urea (Analar, BDH, England) was twice recrystallized from ethanol before use. *N*-Hydroxymethylphthalimide was prepared according to Pucher and Johnson (1922). *N*-Chloromethylphthalimide was prepared according to Sachs (1898). Glycine *N*-phthalimidomethyl ester *p*-toluenesulfonate was prepared according to Nefkens *et al.* (1963). All other reagents were of analytical grade.

N-Benzyloxycarbonyl-L-alanylglycine Phthalimidomethyl Ester (I). Dicyclohexylamine (3.6 g, 20 mmoles) was added to a solution of *N*-benzyloxycarbonyl-L-alanylglycine (Stein *et al.*, 1944) (5.6 g, 20 mmoles) in dimethylformamide (25 ml). After addition of *N*-chloromethylphthalimide (4 g, 21 mmoles) the solution was kept for 5 min at 65° and for an additional 45 min at room temperature. Ethyl acetate (100 ml) was added and the precipitate of dicyclohexylammomium chloride was filtered off and washed with ethyl acetate. The filtrate was washed successively with water, and 5% sodium bicarbonate, and dried over sodium sulfate. The solvent was evaporated to dryness and the residue was crystallized from ethyl acetate, yield 7.2 g (81%), mp 158–159°, λ_{max}^{DMF} 294 mμ (ε 2250).

Anal. Calcd for $C_{22}H_{21}N_3O_7$: C, 60.13; H, 4.82; N, 9.56. Found: C, 60.28; H, 5.01; N, 9.49.

L-Alanylglycine Phthalimidomethyl Ester p-Toluenesulfonate (II). Compound I (8.8 g, 20 mmoles) was suspended in a solution of *p*-toluenesulfonic acid (4 g, 21 mmoles) in methanol (250 ml), and was hydrogenated for 4 hr in a Parr apparatus in the presence of palladium on charcoal (1 g). After filtering off the catalyst, the solution was evaporated to dryness and crystallized from isopropyl alcohol or ethanol–ether, yield 7.65 g (80%), mp 180°, λ_{max} 298 mμ (ε 2500).

Anal. Calcd for $C_{21}H_{23}N_3O_8S$: C, 52.83; H, 4.86; N, 8.80. Found: C, 52.71; H, 4.85; N, 8.57.

Modified RNase Preparations

RNase-glycine.[2] Glycine phthalimidomethyl ester *p*-toluenesulfonate (4 g in 10 ml of water) was added to a solution of RNase (200 mg) in 2 ml of water at room temperature. The solution was brought to pH 4.8 by the addition of 1 N NaOH and WSC[2] (600 mg) was added in two portions under stirring. The reaction was allowed to proceed at room temperature for 4 hr and then the solution was dialyzed in the cold against six changes of distilled water in heated dialysis tubings (Kupke, 1961), and lyophilized. RNase-glycine was obtained by the action of aqueous piperidine on the RNase-glycine phthalimidomethyl ester. The protected RNase modification (200 mg) was suspended at 0° in 0.5 M aqueous piperidine (28 ml) and left for 30 hr at 4° with stirring. The resulting solution was then neutralized by the slow addition of cold acetic acid, exhaustively dialyzed against distilled water, and lyophilized. The progress of the hydrolysis of the phthalimidomethyl ester was followed spectrophotometrically at 299 mμ (Wilchek *et al.*, 1966).

RNase-AlaGly.[2] RNase-AlaGly "A" was prepared from 130 mg of RNase, 2.4 g of II in 15 ml of water, and 800 mg of WSC. The reaction proceeded for 5 hr and the reaction product was treated as described above for RNase-glycine. RNase-AlaGly "B" was prepared from 130 mg of RNase, 3.2 g of II in 15 ml of water, and 240 mg of WSC. In this case the reaction was allowed to proceed for 3 hr.[3]

Methods

Amino acid analysis was conducted in a Spinco Model 120 B automatic amino acid analyzer (Spackman *et al.*, 1958). All hydrolyses were carried out in 6 N HCl in sealed, evacuated ampoules for 22 hr at 116°. The amino acid composition of RNase and its derivatives was calculated by assuming that the hydrolysate contained the theoretical number (Hirs *et al.*, 1956; Smyth *et al.*, 1962; Potts *et al.*, 1962) of 12 alanine residues and 12 glutamic acid residues in the case of RNase-glycine, and 12 glutamic acid residues and 15 aspartic acid residues in the case of RNase-AlaGly. The correction factor used by Gundlach *et al.* (1959) and by Rupley and Scheraga (1963) was employed for tyrosine.

Hydrazinolysis was performed by the method of Akabori *et al.* (1952).

[1] In the nomenclature of peptides the expression "glycyl peptide" designates a peptide in which glycine is bound through its carboxyl group, whereas "glycine peptide" designates a peptide in which glycine is bound through its amino group. In analogy, we suggest designating a derivative in which glycines are bound through their carboxyls to a protein as "glycyl-protein," and a derivative in which glycines are bound to a protein through their amino groups as "protein-glycine." In accord with this suggestion we denote the derivative in which glycine residues are bound to the carboxyl groups of RNase as RNase-glycine.

[2] Abbreviations used in this paper: RNase-glycine, an RNase derivative in which 11 glycine residues are attached to carboxyl groups; RNase-AlaGly, an RNase derivative in which eight L-alanylglycine peptides are attached to carboxyl groups; WSC, water soluble carbodiimide; DMF, dimethylformamide.

[3] The excess of L-alanylglycine phthalimidomethyl ester was recovered from the diffusate, after dialysis, by reaction with carbobenzoxy chloride, followed by the extraction with ethyl acetate of the *N*-carbobenzoxy-L-alanylglycine phthalimidomethyl ester formed.

Desamination was performed according to Anfinsen *et al.* (1962).

Electrophoresis was carried out on cellulose acetate strips (Oxo, England) in 0.05 M sodium acetate buffer, pH 5.0. A voltage of 12 v/cm was applied for 75 min at room temperature.

Spectrophotometric Studies. Ultraviolet absorption spectra were recorded on a Cary 14 automatic double-beam recording spectrophotometer, fitted with thermospacers controlled from an external water-circulation thermostat. Spectrophotometric titrations at 295 mμ were carried out at 25° on a Zeiss PMQ II spectrophotometer in 10-mm quartz cuvets. The pH was measured in a Radiometer TTT1 automatic titrator. The pH was increased by addition of small aliquots of 1 N NaOH from an Agla precision syringe. The proteins were dissolved in deionized water, so that ionic strength increased during the titration with addition of alkali. The concentration of all protein solutions was determined from the optical density at the wavelength of maximal absorption at pH 7.0 (276 mμ for RNase-glycine and 277.5 mμ for RNase), assuming the molar extinction to be ε 9780 (Hermans and Scheraga, 1961). The absorption values obtained during titrations were corrected for dilution. One sample of each protein solution was brought directly to pH 13 with 1 N NaOH and its optical density at 295 mμ was measured after 45 min. The value thus obtained represents the last point of each titration curve.

Assays of enzymic activity of the derivatives and of unmodified RNase were performed on RNA as well as on cytidine 2′,3′-cyclic phosphate as substrates. In the first instance the assay was carried out according to the method of Kunitz (1946), whereas in the second the changes in the optical density of the reaction mixture in 0.1 M Tris–HCl buffer, pH 7.0, were measured at 290 mμ, using quartz cuvets fitted with "inserts" (Pyrocell) to reduce their optical pathway to 0.5 mm. Both assays were performed on the Cary 14 spectrophotometer using the scale-expanding slide wire. Cuvets and reagents were kept at 26°. Enzyme concentrations were determined by optical density measurements as detailed above.

Reduction and reoxidation of RNase and its derivatives was carried out at a concentration of 20 μg/ml for 4 hr according to Anfinsen and Haber (1960).

Results

Preparation and Characterization of RNase-Glycine. RNase-glycine was prepared by the reaction of RNase with an excess of glycine phthalimidomethyl ester in the presence of WSC at pH 4.8. The *N*-phthalimidomethyl groups were removed from the product with 0.5 M piperidine and the excess of unreacted reagents was separated off by exhaustive dialysis. The RNase-glycine preparation thus obtained moved upon electrophoresis on cellulose acetate at pH 5.0 as a single homogeneous spot with mobility somewhat lower than that of the native protein run simultaneously, and displayed no detectable contamination with unmodified RNase.

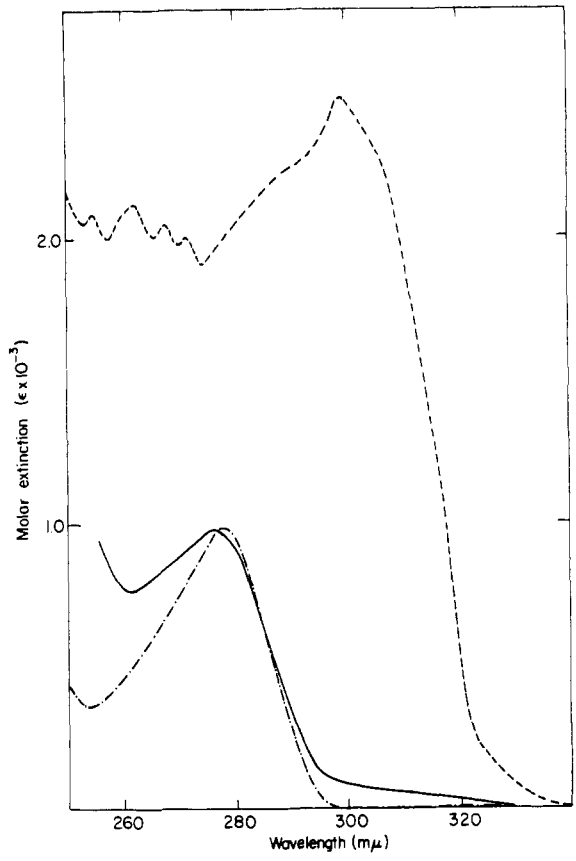


FIGURE 1: Ultraviolet absorption spectra of RNase-glycine (———), RNase-glycine phthalimidomethyl ester (·····), and native RNase (–·–·–·) in water at neutral pH.

The RNase-glycine preparation had the same amino acid composition as unmodified RNase except that it contained 13.6 glycine residues/molecule, corresponding to an enrichment with 11 glycines. This is in agreement with the presence of 11 carboxyl groups/RNase molecule (Hirs *et al.*, 1956; Smyth *et al.*, 1962; Potts *et al.*, 1962), and suggests that all the carboxyl groups of RNase have reacted. An independent evaluation of the number of glycine residues attached per RNase molecule was obtained from the estimation of *N*-phthalimidomethyl groups in the intermediate RNase-glycine phthalimidomethyl ester. Eleven phthalimidomethyl groups per RNase molecule were calculated from the absorbancy at 299 mμ (Wilchek *et al.*, 1966). The ultraviolet spectrum of RNase-glycine phthalimidomethyl ester is given in Figure 1. Hydrazinolysis (Akabori *et al.*, 1952) confirmed that all the carboxyl groups of the original RNase (glutamic acid, aspartic acid, and the C-terminal valine) reacted, as only glycine was detected after this treatment.

As the reaction was performed at pH 4.8 the amino groups of RNase were not expected to react with carboxyl groups, causing possible cross-linking. Indeed,

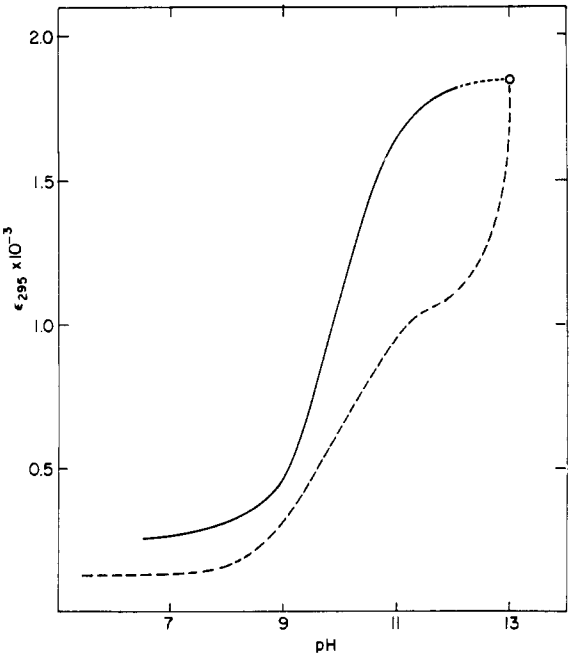


FIGURE 2: Spectrophotometric titration curves of RNase-glycine (———) and of native RNase (– – – –) at 295 mμ and 25°. The protein was dissolved in deionized water and ionic strength increased with addition of alkali. Optical density readings were corrected for dilution. pH values were measured up to 12.5. The last point of each curve was obtained by measuring the optical density of an aliquot 45 min after addition of 1 N NaOH to pH 13.0.

upon complete desamination (Anfinsen *et al.*, 1962), only 0.5 residue of lysine/RNase molecule was found. The same value was obtained after desamination of unmodified RNase.

Spectral Properties of RNase-Glycine. A comparison of the ultraviolet absorption spectra of unmodified RNase and of RNase-glycine revealed a shift of the wavelength of maximal absorption from 277.5 to 276 mμ (Figure 1). Such a shift usually reflects a change in the environment of the phenolic groups of RNase (Scheraga and Rupley, 1962). In view of this observation it was of interest to titrate RNase-glycine spectrophotometrically and find out whether the three tyrosines, known to be "buried" in the native molecule, persist in their unavailability. The spectrophotometric titration curves of unmodified RNase and of RNase-glycine at 25° are given in Figure 2. The titration curve of native RNase exhibits the abnormal titration behavior of three out of six of the tyrosine residues (Shugar, 1952; Tanford *et al.*, 1955). RNase-glycine, on the other hand, displays a normal behavior of all the tyrosine residues in the molecule.

Enzymic Activity. The enzymic activity of the preparation of RNase-glycine *N*-phthalimidomethyl ester and of RNase-glycine was determined using as substrates both RNA (at pH 5.0) and the low molecular weight cytidine 2′,3′-cyclic phosphate. RNase-glycine *N*-phthalimidomethyl ester was completely inactive when assayed with either of these substrates. RNase-glycine, on the other hand, was totally inactive on RNA but it had 93% of the activity of unmodified RNase when assayed on cytidine 2′,3′-cyclic phosphate.

Reduction and Reoxidation of RNase-Glycine. The modified enzyme was reduced in 8 M urea with β-mercaptoethanol (Anfinsen and Haber, 1960). The reaction product was freed from urea and β-mercaptoethanol by passage on Sephadex G-25 in 0.1 M acetic acid and assayed for activity on cytidine 2′,3′-cyclic phosphate. The reduced derivative was completely inactive. Full initial activity was restored upon air reoxidation at a concentration of 20 μg/ml.

RNase-AlaGly. Two RNase-AlaGly derivatives were prepared, similarly to RNase-glycine, by the reaction of RNase with an excess of II and WSC, and the removal of the protecting groups with piperidine. The amino acid analysis of these derivatives revealed an enrichment with eight and seven L-alanylglycine dipeptides, respectively, for derivatives "A" and "B." Upon desamination, 0.5 residue of lysine/molecule was found, suggesting that no side-chain reactions involving amino groups of lysine have occurred. As the hydrazinolysis revealed glycine but no valine it may be concluded that the C-terminal carboxyl of valine was substituted with alanylglycine. The RNase-AlaGly preparations, as well as their phthalimidomethyl intermediates, were completely inactive when assayed on either RNA or on cytidine 2′,3′-cyclic phosphate.

Discussion

The experiments described here indicate that it is possible to bind amino acids or peptides to the carboxyl functions of proteins, making use of a reversible blocking group for the amino acid or peptide carboxyl groups and of a water-soluble carbodiimide for the coupling reaction. Carbodiimides have been used previously to bind small molecules to synthetic polypeptides (Sela *et al.*, 1964; Haber *et al.*, 1965) and to proteins (Goodfriend *et al.*, 1964; Halloran and Parker, 1966). The esterification of the carboxyl groups has been among the earliest modifications of functional groups in proteins (for review articles, *cf.* Herriot, 1947 and Sri Ram *et al.*, 1962). Hoare and Koshland (1966) have shown recently that it is possible to bind amino acid esters to the carboxyl groups of proteins by making use of a water-soluble carbodiimide. In order to modify the carboxyl groups of RNase, Riehm and Scheraga (1966) have used the direct reaction of RNase with a WSC.

In the present study we were interested in modifying the carboxyl groups of RNase without changing the electrical charges due to the carboxylate ions. For this purpose the protein was treated either with glycine phthalimidomethyl ester or with L-alanylglycine phthalimidomethyl ester in the presence of WSC, and the phthalimidomethyl groups were removed from the resulting modified RNase preparations with 0.5 M

piperidine. Previous work from this laboratory (Wilchek *et al.*, 1966) has shown that these conditions do not interfere with the activity of RNase and that the phthalimidomethyl group was a useful, reversible blocking reagent for the γ carboxyls of glutamate residues in the synthesis of poly-L-glutamic acid and poly-L-glutamyl proteins. The phthalimidomethyl group has also been used successfully for the blocking of the carboxyl functions of RNase, followed by the removal of the blocking groups and concomitant recovery of enzymic activity (B. S. Wildi and C. B. Anfinsen, personal communication).

The esterification with methanol of all (Sela *et al.*, 1957) or most (Broomfield *et al.*, 1965; Riehm *et al.*, 1965) of the carboxyl groups of RNase resulted in derivatives devoid of enzymic activity on RNA. Similarly, the RNase-glycine phthalimidomethyl ester preparation described here is devoid of activity toward RNA, as well as the low molecular weight substrate cytidine 2',3'-cyclic phosphate. The removal of the phthalimidomethyl groups from this preparation resulted in the recovery of most of the activity of the native enzyme toward cytidine 2',3'-cyclic phosphate but not toward RNA. It thus seems that the restoration of the electrical charges was instrumental in the recovery of activity. Nevertheless, the precise steric position of the carboxylate ions in RNase seems to play a role in the enzymic activity, as RNase-glycine acts only on the low molecular weight substrate but not on RNA. Moreover, RNase-AlaGly preparations, in which the carboxylate ions are even further removed from the original positions of the RNase carboxyls, are not active at all on either substrate.

The reduction of the disulfide bridges of RNase followed by their reoxidation results in the re-formation of the native molecule possessing all its original enzymic activity (Anfinsen and Haber, 1960). Similarly, most enzymically active chemical derivatives of RNase, including polypeptidyl RNases, were shown to recover their full initial activity after reduction and reoxidation (Epstein and Goldberger, 1964; Anfinsen *et al.*, 1962; Wilchek *et al.*, 1966). RNase-glycine behaved in the same way, *i.e.*, it lost all its activity on cytidine 2',3'-cyclic phosphate upon reduction, and recovered all this activity after reoxidation. This finding establishes, *inter alia*, that the catalytic activity of RNase-glycine on cytidine 2',3'-cyclic phosphate is indeed a property of the RNase molecule related to its conformation and not a new hydrolytic activity resulting from the attachment of glycine to the β-carboxyl of aspartic acid or to the γ-carboxyl of glutamic acid. RNase-glycine is not unique in the feature of being active on the low molecular weight substrate only. Poly-L-ornithyl-RNase and poly-L-arginyl-RNase are similarly active on cytidine 2',3'-cyclic phosphate but completely inactive toward RNA in the whole pH range in which native RNase is active (A. Frensdorff, M. Wilchek, and M. Sela, 1966, unpublished data).

The ultraviolet absorption spectrum of fully methylated RNase resembled that of unfolded RNase derivatives in that its maximum adsorption was shifted to a shorter wavelength (Sela *et al.*, 1957). On the other hand, a RNase derivative in which eight out of eleven carboxyls per molecule were methylated exhibited a spectrum similar to that of native RNase (Broomfield *et al.*, 1965). As seen in Figure 1, RNase-glycine, a derivative in which all the carboxyls of RNase were modified, exhibits a maximum of absorption at 276 mμ, to be compared to 277.5 mμ for native RNase.

The spectrophotometric titration curve of this derivative is also different from that of native RNase, in which three tyrosine residues are "buried" (Shugar, 1952; Tanford *et al.*, 1955). As seen in Figure 2 all the tyrosines are available for ionization in RNase-glycine. It is known that some carboxyl groups in native RNase are involved in specific tyrosine–carboxylate interactions (Hermans and Scheraga 1961; Riehm and Scheraga, 1966), and their location in the amino acid sequence of the enzyme has recently been elucidated (Li *et al.*, 1966). As in RNase-glycine the carboxylate ions have not been abolished but only displaced, it seems that the precise spatial position of some carboxyl groups in RNase is crucial for the formation of the correct tyrosine–carboxylate bonds. Generally, enzymically active derivatives of RNase have exhibited a spectrum similar to native RNase (Sela *et al.*, 1957; Scheraga and Rupley, 1962). The partially methylated RNase derivative described by Broomfield *et al.* (1965) exhibited a "native" spectrum, even though devoid of enzymic activity. The RNase-glycine preparation described here has a shifted spectrum and is devoid of activity on RNA. Nevertheless, it is almost as active as the native enzyme when cytidine 2',3'-cyclic phosphate is used as substrate. In view of this observation it is possible that other RNase derivatives described in the literature (which were assayed only on RNA as substrate) may be active on pyrimidine 2',3'-cyclic phosphates even though inactive on RNA and exhibiting an "unfolded" ultraviolet absorption spectrum.

The method described here for the attachment of free amino acids and peptides to the carboxyl groups of proteins may be of interest for the elucidation of the role of electrical charges and their precise positions within the molecule, both in conformation and in the biological activity of a protein. Moreover, it may permit the attachment of peptides of defined sequence to predetermined positions in the protein molecule.

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