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# The Reversible Masking of Amino Groups in Ribonuclease and Its Possible Usefulness in the Synthesis of the Protein

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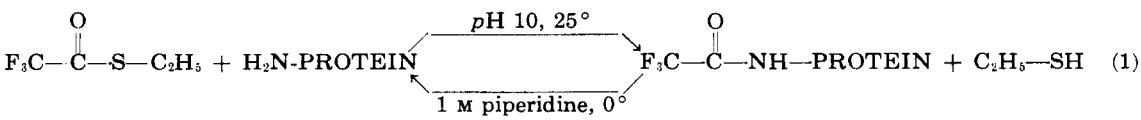
In the present report a method is described by which the amino groups of ribonuclease can be trifluoroacetylated by reaction with ethyl thioltrifluoroacetate. By this method the lysyl bonds in the protein molecule are rendered resistant to hydrolysis by trypsin, while the arginyl bonds are left susceptible. The amino acid compositions of the five peptides isolated from a tryptic digest of trifluoroacetylated ribonuclease (in its oxidized form) were shown to correspond with the known compositions of the segments of ribonuclease expected upon cleavage of arginyl bonds. The trifluoroacetyl groups can be removed from the trifluoroacetylated enzyme by exposure to 1.0 M piperidine at 0°. The material thus obtained lacks enzymic activity, presumably owing to the presence of incorrectly paired half-cystine residues. Rearrangement of disulfide bonds, under conditions known to favor the assumption of "native" configuration, leads to the regeneration of full enzymic activity. The method for the reversible masking of amino groups is discussed in relation to its possible usefulness in the organic synthesis of ribonuclease.

The availability of the complete formula for the covalent structure of bovine pancreatic ribonuclease (RNase),[1] together with an extensive body of knowledge concerning its chemical and enzymic properties, make it reasonable to undertake the "organic" synthesis of this enzyme. Such a project is feasible only because the last step in the over-all synthesis, the correct formation of four disulfide bonds and the assumption of native tertiary structure, takes place spontaneously during air oxidation of the reduced polypeptide chain (Anfinsen and Haber, 1961; White, 1961; Anfinsen *et al.*, 1961).

The stepwise synthesis of RNase by methods such as those employed by Hofmann and his colleagues (Hofmann, 1960) for adrenocorticotropic hormone appears to be beyond the possibilities afforded by presently available techniques. However, these methods might ultimately be applicable to segments of the polypeptide chain. A prerequisite for total synthesis is the demonstration that such segments can be isolated from partial enzymic digests and subsequently rejoined in proper sequence with restoration of enzymic activity. The present paper reports on the masking of the amino groups of the protein by reaction with ethyl thioltrifluoroacetate (Schallenberg and Calvin, 1955), as shown in equation (1). The resulting trifluoroacetyl-enzyme (TFA-RNase), both in its intact form and after conversion to the extended polypeptide chain by oxidation or reduction of disulfide bonds, is cleaved by trypsin specifically at the four arginyl bonds in the molecule. The specificity of this cleavage is the result of the modification of ε-amino groups



(1)

[1] Abbreviations: Ribonuclease (RNase); oxidized ribonuclease (Ribox); trifluoroacetyl (TFA); trifluoroacetylated ribonuclease (TFA-RNase); trifluoroacetylated oxidized ribonuclease (TFA-Ribox). Abbreviations for individual amino acids are as recommended by Brand and Edsall (1947).

of the protein, which renders the lysyl bonds resistant to hydrolysis by trypsin, just as after reaction with dinitrofluorobenzene (Redfield and Anfinsen, 1956), carbobenzoxy chloride (Anfinsen *et al.*, 1956), or carbon disulfide (Merigan *et al.*, in press). The trifluoroacetyl derivative is of unique value for this purpose because of its intermediate stability to spontaneous hydrolysis.

The trifluoroacetyl groups could be removed from the protein by treatment with piperidine. Full enzymic activity could be regenerated by reduction and air oxidation of this material.

## Experimental Procedure

*Materials and Analytical Methods.*—Chromatographic grade RNase (Lots #R316-214), was obtained from Sigma Chemical Company. Ethyl thioltrifluoroacetate (ET-TFA) was prepared by the procedure of Hauptschein *et al.* (1952). Piperidine (Fisher Scientific Company), was distilled over KOH and used as a 1.0 M aqueous solution. Dialysis tubing was heated at 80° for 84 hours to diminish its permeability to RNase (Kupke, 1961). Crystalline trypsin (Lot #TR-SF-789), was obtained from Worthington Biochemical Corporation. Tryptic digestions were performed with the *p*H maintained at 8.0 in a *p*H-stat equipped with a Radiometer TTTla autotitrator and a drum recorder manufactured by Ole Dich, Copenhagen; the amount of trypsin used was equal to 1% that of the substrate (by weight). The number of peptide bonds split by trypsin was calculated from the amount of base required to maintain the *p*H at 8.00. In the calculations a *pK* of 7.8 was assumed for the $\alpha$-amino groups released. All centrifugations were performed at 3° in a Servall centrifuge, Model SS-1, at 10,000 rpm. Protein concentrations of RNase solutions were determined by calculation from the absorption at 280 m$\mu$ and the known extinction of RNase, with a Beckman DU spectrophotometer. Absorption spectra were recorded with a Cary Spectrophotometer, Model 14, using quartz cuvets with a 10-mm light path. Assays for the enzymic activity of RNase were performed by the procedure of Anfinsen *et al.* (1954). Determination of amino end-groups by reaction with dinitrofluorobenzene was performed by a modification (Redfield and Anfinsen, 1956), of the method of Levy (1954). Gel filtration was carried out at room temperature on columns of Sephadex G-25 (Pharmacia Company, Uppsala, Sweden), equilibrated with 0.1 N acetic acid. Acid hydrolysis of peptides was carried out in sealed, evacuated tubes with 6.0 N HCl at 110° for 24 hours. Amino acid analysis of peptide hydrolysates was performed by the method of Dreyer (1960) and of Dreyer and Bynum (in press). Oxidized RNase (Ribox) was prepared by performic acid treatment of RNase as previously described (Hirs, 1956; Redfield and Anfinsen, 1956).

*Preparation of Trifluoroacetylated RNase.*—In a 125-ml Erlenmeyer flask 0.5 g of RNase was dissolved in 50 ml of water. The flask was placed on a magnetic stirrer, and the electrode of a *p*H meter was immersed in the solution. The *p*H was raised to 10.0 by the addition of 1.0 N KOH. The base was added from a syringe fitted with a No. 19 needle attached to polyethylene tubing. Ethyl thioltrifluoroacetate (2.5 ml) was added to the solution. This reagent, immiscible in water, was divided into small droplets by the revolving magnet, exposing a large surface area for reaction with protein in the aqueous phase. The *p*H was maintained between 9.95 and 10.00 by the addition of KOH from the syringe. The reaction was carried out at room temperature and was allowed to proceed for about 1 hour, after which time the base uptake (approximately 18 ml) had essentially ceased. A large amount of alkali was required because of the rapid spontaneous hydrolysis of ethyl thioltrifluoroacetate. To the reaction mixture was added 5.0 ml of 0.1 M sodium acetate buffer, *p*H 5.0. The *p*H was lowered to 6.0 by the addition of a few drops of concentrated HCl, and a copious white precipitate was obtained. The reaction mixture was added to 4 volumes of cold (−20°) absolute ethanol. The precipitate was collected by centrifugation and washed twice with a mixture of ethanol and sodium acetate buffer (5:1 by volume) at 3°. The final residue was suspended in 50 ml of the ethanol sodium acetate mixture and dialyzed against 100 volumes of distilled water at 3° for 24 hours (with 6 changes of dialysis fluid). The contents of the dialysis sac were centrifuged and the supernatant fluid, containing a few milligrams of protein, was discarded. The residue was dried by lyophilization and was found to represent about 75% of the theoretical yield of TFA-RNase.

*Removal of Trifluoroacetyl Groups from TFA-RNase.*—Ten mg of TFA-RNase was dissolved in 0.5 ml of 1.0 M piperidine at room temperature and the solution was immediately chilled in an ice bath. After incubation in the ice bath for 2 hours, the reaction mixture was slowly added, with constant stirring, to 1.5 ml of 0.5 N acetic acid which had been cooled to about 3°. The solution was subjected to gel filtration on a 2.5 × 25 cm column of Sephadex. The effluent fluid was collected in 3.0-ml aliquots and, as shown in Figure 1, the protein peak was found to emerge from the column well ahead of the piperidine (which was identified by absorbing contaminants). The fractions containing the protein peak were pooled and lyophilized. The white, fluffy powder thus obtained represented about 60% of the theoretical yield of RNase.

## Results

*Properties of TFA-RNase.*—In aqueous media, TFA-RNase was extremely insoluble in the *p*H range below 6.0 and only slightly soluble between 6.0 and 7.5. The trifluoroacetyl groups were

stable to 0.03 N HCl in acetone at 3° for 2 hours and to *p*H 9.0 at 40° for 1 hour, but in the *p*H range above 10.0 they became labile. The absorption maximum of TFA-RNase was at 276.0 mμ. It was not active enzymically, nor was activity restored by application of the standard methods for reduction of its disulfide bonds and reoxidation in air. No free amino groups could be detected by reaction with dinitrofluorobenzene, indicating that the ε-amino groups of the lysine residues and the terminal α-amino group had all been masked. Digestion of TFA-RNase with trypsin was attended by the uptake of an amount of base corresponding (within the limits of experimental error) to the splitting of four peptide bonds.

*Properties of Material Produced on Hydrolysis of TFA-RNase by Piperidine.*—The material produced on hydrolysis of TFA-RNase by piperidine displayed solubility characteristics similar to those of native RNase. By treatment of this material with dinitrofluorobenzene, the number of free amino groups was found to be the same as for the native enzyme. Its absorption maximum, however, was at 275.8 mμ, and its enzymic activity was only about 0.2% that of native RNase. Unlike the native enzyme, which is resistant to hydrolysis by trypsin at 30°, this material on tryptic digestion took up an amount of base corresponding to the splitting of approximately twelve peptide bonds.

*Regeneration of Native RNase.*—The disulfide bonds of the material obtained on hydrolysis of TFA-RNase by piperidine were reduced by treatment with 2-mercaptoethanol in the presence of 8.0 M urea, and subsequently allowed to reoxidize in air by the procedure of Anfinsen and Haber (1961). Like native RNase, the material thus obtained had an absorption maximum at 277.5 mμ and was resistant to hydrolysis by trypsin. The enzymic activity of this material was 80–100% that of the native RNase used as starting material. Amino acid analyses gave results identical with those for the native enzyme.

*Tryptic Digestion of TFA-Ribox.*—TFA-Ribox, prepared from Ribox by the same method as that used for native RNase, was subjected to digestion by trypsin. The amount of base consumed during the digestion corresponded to the splitting of four peptide bonds. Analysis of an aliquot of the reaction mixture for free amino end-groups disclosed the presence of glutamic acid, aspartic acid, and cysteic acid in the ratio 2:1:1, precisely the result expected upon cleavage of the four arginyl bonds in the molecule (Anfinsen *et al.*, 1956; Redfield and Anfinsen, 1956). No other end-groups were observed.

*Characterization of Peptides from Trypsin-Digested TFA-Ribox.*—After digestion of TFA-Ribox with trypsin the entire reaction mixture was lyophilized. The TFA groups were removed by treatment of the dry powder with piperidine under the same conditions as those described above for TFA-RNase. After acidification with acetic acid the reaction mixture was passed through a 2.5 × 50 cm column of Sephadex G-25 equilibrated with 0.1 N acetic acid. A graph in which effluent volume is plotted against optical density is shown in Figure 2. The material obtained from the column was pooled into four fractions (1, 2, 3, and 4). After lyophilization, the fractions were dissolved in 0.5 ml of water and applied to filter paper. Separation of the peptides in each fraction was accomplished by electrophoresis in pyridine-acetate buffer, *p*H 6.5, under the conditions previously described (Katz *et al.*, 1959). As shown in Figure 3, only five peptides (*a*, *b*, *c*, *d*, and *e*) were found to be present. Some of the peptides were located in more than one fraction, owing to the poor separation on Sephadex (*i.e.*, peptides *d* and *e* were found in fraction 1; *a*, *b*, and *d* in fraction 2; *a*, *b*, and *c* in fraction 3; and *c* alone in fraction 4). Zones corresponding to the individual peptides were cut out of the dried electrophoresis papers, eluted with water and lyophilized. After hydrolysis with HCl, their amino acid compositions were determined by the technique of Dreyer (1960) and of Dreyer and Bynum (1962). The results of these analyses are shown in Table I, together with the known amino acid compositions of the peptides expected upon cleavage of the four arginyl bonds in RNase.

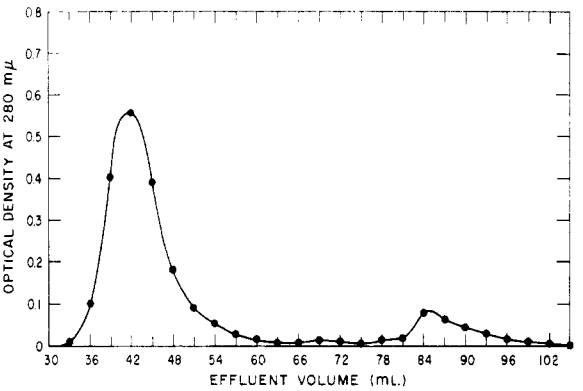



FIG. 1.—Separation of protein from reagents by gel filtration, following treatment of TFA-RNase with piperidine. After incubation of TFA-RNase with piperidine the reaction mixture was acidified with 0.5 N acetic acid and subjected to gel filtration on Sephadex G-25, with 0.1 N acetic acid as solvent. The effluent fluid was collected in 3.0-ml fractions and the optical density at 280 mμ determined. The first peak, corresponding to protein, was well separated from the second, which represents the reagents (trifluoroacetate and piperidine).

## DISCUSSION

The material prepared by removal of trifluoroacetyl groups from TFA-RNase by treatment with piperidine resembled native RNase in amino acid composition, in solubility, and in its content of free amino groups. It differed from the native

TABLE I

AMINO ACID COMPOSITION OF PEPTIDES PREPARED BY TRYPTIC DIGESTION OF TRIFLUOROACETYLATED RIBOX

| | Fraction *a* (found) | Residues 1–10 (theor) | Fraction *b* (found) | Residues 11–33 (theor) | Fraction *c* (found) | Residues 34–39 (theor) | Fraction *d* (found) | Residues 40–85 (theor) | Fraction *e* (found) | Residues 86–124 (theor) |
|---|---|---|---|---|---|---|---|---|---|---|
| Lys | 2+ | 2 | 2+ | 1 | 3+ | 1 | 2+ | 3 | 4+ | 3 |
| His | | | 2+ | 1 | | | 1+ | 1 | 3+ | 2 |
| Arg | 1+ | 1 | 1+ | 1 | 2+ | 1 | 1+ | 1 | | |
| Asp | | | 2+ | 3 | 3+ | 2 | 4+ | 6 | 4+ | 4 |
| Thr | 1+ | 1 | | | 2+ | 1 | 3+ | 4 | 3+ | 3 |
| Ser | | | 4+ | 7 | | | 4+ | 5 | 3+ | 3 |
| Glu | 2+ | 2 | 2+ | 2 | | | 3+ | 5 | 3+ | 3 |
| Pro | | | | | | | 1+ | 1 | 3+ | 3 |
| Gly | | | | | | | 1+ | 1 | 3+ | 2 |
| Ala | 3+ | 3 | 1+ | 2 | | | 2+ | 3 | 3+ | 4 |
| Val | | | | | | | 4+ | 5 | 3+ | 4 |
| Ileu | | | | | | | 1+ | 1 | 2+ | 2 |
| Leu | | | | | 2+ | 1 | 1+ | 1 | | |
| Tyr | | | 2+ | 2 | | | 2+ | 2 | 3+ | 3 |
| Phe | 1+ | 1 | | | | | 2+ | 1 | 2+ | 1 |

Amino acid compositions for peptides *a–e*, isolated from a tryptic digest of TFA-Ribox, were determined by electrophoresis on paper (Dreyer, 1960; Dreyer and Bynum, in press). The relative amounts of the individual amino acids were rated 1+–4+ on the basis of the color intensity of the spots on paper as compared with standards. The known amino acid composition (in residues/mole), of each of the five peptides that would, theoretically, be obtained from Ribox by cleavage of the four arginyl bonds appears immediately to the right of the corresponding peptide actually isolated. No special attempt was made to estimate cysteic acid or methionine sulfone, since identification of the peptides was possible without these data.

enzyme, however, in its susceptibility to hydrolysis by trypsin, in the position of its absorption maximum (275.8 mμ), and in its lack of enzymic activity. All the above characteristics have been observed previously for derivatives of RNase in which the pairing of half-cystine residues differs from that of the native molecule (Haber and Anfinsen, in press). The hypothesis that the material prepared by incubation of TFA-RNase with piperidine contained incorrect pairing of half-cystine residues was supported by the finding that reduction in the presence of urea, followed by reoxidation in air (a procedure thought to lead to the formation of the correct disulfide bonds [White, 1961; Anfinsen *et al.*, 1961]), led to a shift in the absorption maximum to 277.5 mμ (that of native RNase), to resistance to tryptic digestion, and to complete restoration of enzymic activity. In the present experiments the stage at which disulfide interchange probably occurred was during reaction of RNase with ethyl thioltrifluoroacetate. The presence of ethane thiol, released during the reaction, at alkaline *p*H values would clearly favor such interchange.

Experiments on tryptic digestion of TFA-Ribox and characterization of the resulting peptides demonstrate the usefulness of trifluoroacetylation as a technique for limiting the trypsin susceptibility of a protein to arginyl bonds. Although our investigations have been carried out only on RNase, it appears likely that the method will be useful in studies of covalent structure in other proteins.

It is a well-established principle in peptide chemistry that the synthesis of a polypeptide chain from two fragments requires the reversible blocking of all reactive functional groups other than the amino and carboxyl groups to be joined through a peptide bond. The reversible blocking of the amino groups in RNase is now possible through the use of trifluoroacetylation. Investigations on an analogous technique for carboxyl groups are in progress. The reversible stabilization of sulfhydryl groups produced by reduction

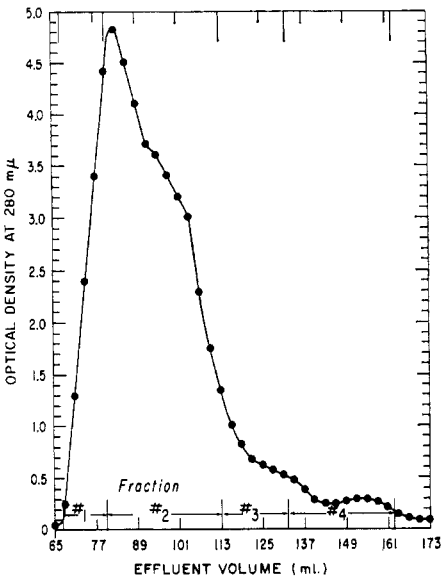



FIG. 2.—Gel filtration of a tryptic digest of TFA-Ribox. After tryptic digestion of TFA-Ribox the trifluoroacetyl groups were removed by treatment with piperidine, and the entire reaction mixture passed through a 2.5 × 50 cm column of Sephadex G-25, with 0.1 N acetic acid as solvent. The effluent volume is plotted against absorption at 280 mμ. The effluent fluid was pooled into four fractions, as shown near the bottom of the graph.

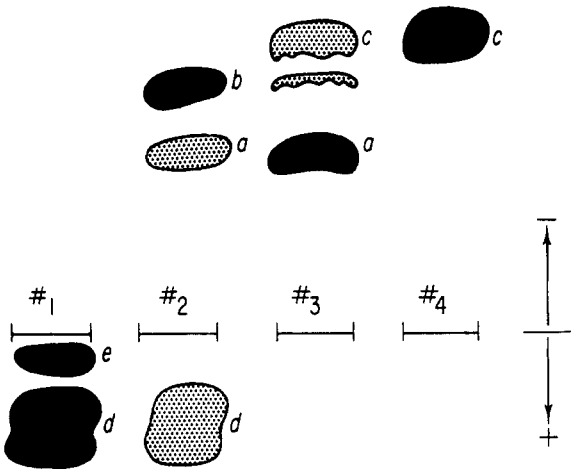



FIG. 3.—Electrophoresis of the four fractions obtained by gel filtration of trypsin-digested TFA-Ribox. After lyophilization, the four fractions were dissolved in 0.05 ml of water and 10% of the material applied to filter paper to determine the optimal conditions for electrophoresis. Electrophoresis was carried out in pyridine-acetate buffer by the method previously reported (Katz *et al.*, 1959). After 1 hour, five peptides had separated on the paper (the period of electrophoresis for the remaining 90% of the material was increased to 3 hours for the preparative work described in the text). The fraction numbers (which correspond to those in Figure 2) are shown just above the origin. The order in which the peptides are designated (*a*, *b*, *c*, *d*, and *e*), matches the order of the corresponding peptides of native RNase.

of the cross-linked molecule, and the susceptibility of the stabilized RNase derivative to trypsin digestion, have been discussed in a previous report (Anfinsen and Haber, 1961).

Kinetic studies (Redfield and Anfinsen, 1956) on the trypsin digestion of DNP-Ribox have shown that it is possible to limit hydrolysis to the extent of one to two arginyl bonds. Such limited proteolysis may be expected to yield a restricted population of cleavage products made up, for the most part, of two or three unique peptide fragments. Should the separation and recombination of fragments and subsequent unmasking of carboxyl groups pose no insurmountable problems, the final regeneration of the tertiary structure of the enzyme through oxidation of sulfhydryl groups can be accomplished by the established procedures (White, 1961; Anfinsen *et al.*, 1961).

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