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Acetimidation of Bovine Pancreatic Ribonuclease A*

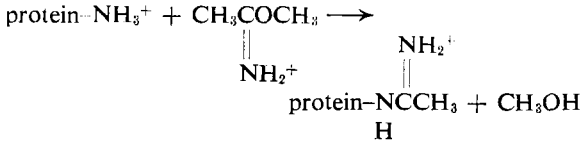
John H. Reynolds

ABSTRACT: All of the amino groups of bovine pancreatic ribonuclease A have been substituted with methyl acetimidate to give an enzymatically inactive protein which resembles ribonuclease A in its physical and chemical properties. The facts that completely acetimidated ribonuclease is not hydrolyzed by trypsin and that its optical rotatory dispersion spectrum, ultraviolet spectrum (ϵ 9900), and sedimentation coefficient ($s^0_{20,w}$ = 2.3 S) are almost identical with that of ribonuclease A indicate that no large structural changes have occurred upon acetimidation. Full ribonuclease activity can be regained by deacetimidation with concentrated aqueous ammonia. The product of this reaction is not chromatographically identical with that of ribonuclease A on SE-Sephadex but the amino acid content is identical with that of the original protein. When the disulfide bonds in completely acetimidated ribonuclease are reduced, reoxidized, and the acetimido groups removed, enzymatic activity is regained. Performic acid oxidized acetimidated ribonuclease is susceptible to trypsin-catalyzed hydrolysis only at arginyl peptide bonds. The five resulting peptides have been identified by paper chromatography, cellulose acetate electrophoresis, and ion-exchange chromatography.

Thus acetimidation can provide a method for selectively blocking the action of trypsin at lysyl bonds while allowing the protein to retain much of its original structure.

Several reports in the recent literature have dealt with the reaction of imido esters with proteins. Insulin (Hunter and Ludwig, 1962) and bovine serum albumin (Wofsy and Singer, 1963; Habeeb, 1966) have been modified with methyl acetimidate and the products were studied in some detail. A review has recently appeared (Ludwig and Hunter, 1967). Hartman and Wold (1967) have reacted RNase with a bifunctional imido ester to produce an intramolecular cross-linked active enzyme. Because our work required a selective reversible modification of lysine amino groups in proteins which would block tryptic digestions we chose to study the reaction of methyl acetimidate with bovine pancreatic RNase A.

We have found that all of the amino groups of RNase react with methyl acetimidate according to the scheme below to produce an inactive protein whose physical and chemical properties are very similar to native RNase. Complete enzymatic activity can be regenerated upon removal of the acetimido groups. Trypsin activity is confined to the arginyl peptide bonds in performic acid oxidized acetimido RNase (AIROX).[1] The details of the chemistry of completely acetimidated ribonuclease (AIR) and its preparation and regeneration to active RNase are reported here.



Experimental Section

Materials. Bovine pancreatic RNase was purchased from Worthington Biochemical Corp. as a three-times-recrystallized crude mixture and also as pure RNase A. The crude material is heterogeneous on SE-Sephadex and contains about 50% impurities and RNase B and 50% RNase A. Methyl acetimidate hydrochloride was prepared from hydrogen chloride, acetonitrile, and methanol according to published procedures (Hunter

* From the Research Center, Monsanto Company, St. Louis, Missouri 63166. *Received April 29, 1968.*

[1] Abbreviations used: RNase, bovine pancreatic ribonuclease; AIR, completely acetimidated RNase; RIBOX, performic acid oxidized RNase; AIROX, performic acid oxidized AIR; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; TNBS, trinitrobenzenesulfonic acid. For other abbreviations, see *Biochemistry 5*, 1445 (1966).

and Ludwig, 1962). TPCK trypsin and yeast RNA were purchased from Worthington.

Methods. Amino acid analyses were performed on the Beckman 120B. Peptide mapping was carried out according to the methods described by Katz *et al.* (1959). Polyacrylamide gel electrophoreses were performed at pH 6.6. Cellulose acetate electrophoresis was run at 400 V for 50 min at 25° in the Gelman high-resolution buffer containing Tris and Veronal (pH 8.8). The peptides on the cellulose acetate strips were identified with Ponceau S stain. The molar extinction coefficient of AIR was measured by weighing a sample which had been dried over phosphorous pentoxide, dissolving it in 0.05 M ammonium acetate (pH 6.8), and recording the spectrum on a Cary 14 spectrophotometer using 1-cm quartz cells. Optical rotatory dispersion spectra of desalted samples of RNase and AIR were determined on a Cary 60 spectropolarimeter at 25°. The sedimentation rate of AIR was determined in 0.1 M KCl for a period of 80 min. Solutions were from 0.5 to 1.5% protein by weight. The sedimentation coefficient was calculated from the data according to Schachman (1957). Paper chromatography was performed in *n*-butyl alcohol–water–acetic acid (4:5:1, v/v, top phase) at 25° for 22 hr in a glass jar saturated with solvent vapor. RNase assays were carried out with RNA according to Anfinsen *et al.* (1954) and with the barium salt of uridine 2′,3′-cyclic monophosphoric acid (Sela, 1967).

The number of lysines blocked upon acetimidation was determined by reaction of the modified protein with TNBS according to the method described by Habeeb (1965) and by reaction with ninhydrin (Greenstein and Winitz, 1961) using the extinction coefficients of Slobodian *et al.* (1962).

The tryptic digest of RIBOX and AIROX was chromatographed on Technicon Chromobeads P (0.9 × 110 cm column) using a citrate–acetate buffer system increasing in ionic strength from 0.2 to 2 N and in pH from 3.1 to 6.5 at 4°. The effluent from the column was hydrolyzed with base and allowed to react with ninhydrin in a Technicon peptide analyzer.

The spectrophotometric titration of AIR was carried out by observing the difference in absorption at 2950 Å of AIR (9.35×10^{-2} M) in 0.15 M KCl at pH 7 in the reference cell and the same solution of AIR in the sample cell but at different pH. The latter was adjusted by adding small amounts of 1, 5, or 10 M KOH so that the change in protein concentration was negligible.

Acetimidation of RNase was carried out at pH 10.0. Methyl acetimidate hydrochloride in 200-fold molar excess was added to aqueous solutions of 0.5% protein. Three additions of the reagent were made at 20-min intervals. The pH was initially adjusted manually with 40% NaOH and then automatically with 1 N HCl using a Radiometer pH-Stat. The final solution was dialyzed against distilled water at 4° and lyophilized.

Deacetimidation was accomplished by dissolving AIR in a solution of concentrated ammonia–acetic acid (30:2, v/v) apparent pH 11.3 according to Ludwig and Byrne (1962). After the desired reaction time the solution was diluted with water and lyophilized.

The disulfide bonds in AIR were reduced and reoxidized according to the method of Anfinsen and Haber (1960). The method of Hirs (1956) was used to oxidize AIR with performic acid to AIROX.

The number of carboxylate groups of the regenerated RNase was determined by the method of Hoare and Koshland (1967), coupling them with glycine methyl ester in the presence of ethyldimethylaminopropyl carbodiimide and determining the number of excess glycine residues after acid hydrolysis by amino acid analysis.

Digestion with Trypsin. AIROX was dissolved in 0.01 M ammonium bicarbonate (pH 7.8) containing 0.001 M calcium chloride. The protein concentration was 0.5%. TPCK trypsin was added to give an enzyme-to-substrate ratio of 1:100. The reaction was allowed to proceed at 37° for 6 hr and the solution was lyophilized prior to peptide analysis. Both RNase and AIR were subjected to the action of trypsin in 10% ammonium bicarbonate at 37° for 24 hr with an enzyme-to-substrate ratio of 1:100. The solutions were then lyophilized and the residue were subjected to peptide mapping.

Results

The molar extinction coefficient of ϵ- and α-TNP-amino groups in proteins has been measured by several workers and is about 1.2×10^4 (Goldfarb, 1966). In the TNBS assay, TNP-RNase has an extinction coefficient of 1.5×10^5 which when divided by the number of ϵ- and α- (11) amino groups gives a slightly larger average (1.3×10^4). The extinction coefficient for AIR is 0.5×10^4 indicating that about $(0.5 \times 10^4)/(1.5 \times 10^5)$

TABLE I: Amino Acid Content of RNase and Derivative.[a]

Amino Acid	AIR	Regenerated RNase	RNase
Lys	3.4	9.5	10.2
His	3.6	3.6	3.6
Arg	4.3	3.6	3.9
Asp	15.5	15.7	15.1
Thr	9.4	9.4	9.0
Ser	13.2	13.0	12.4
Glu	12.8	12.7	11.6
Pro	3.7	3.8	4.0
Gly	4.2	3.8	3.5
Ala	13.0	12.6	12.4
Cys	6.3	5.9	6.5
Val[b]	9.0	9.0	9.0
Met	3.0	3.9	3.3
Ile	2.9	2.5	2.5
Leu	2.9	2.6	2.5
Tyr	4.8	4.6	5.1
Phe	3.0	3.1	2.9

[a] 24-hr hydrolyses, uncorrected for losses. [b] Used as standard.

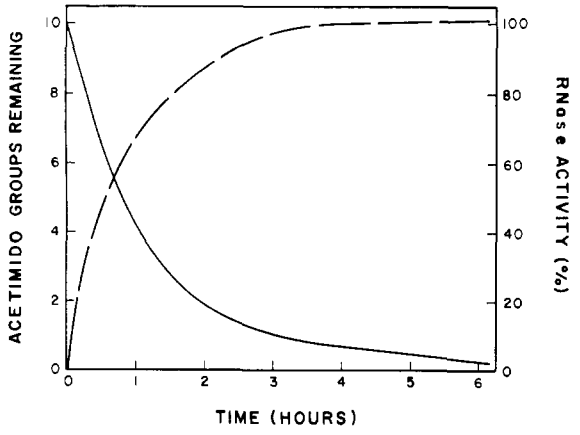


FIGURE 1: Rate of loss of acetimido groups (———) and increase of RNase activity (– – – –) upon ammonolysis of AIR (25°).

× 100) 3% of the amino groups remain unblocked. The same result was obtained from the ninhydrin analysis.

Amino acid analysis of AIR (Table I) showed no changes from that of RNase except for lysine. Acetimidolysine, which is eluted along with NH_3 and therefore cannot be directly determined, is slowly hydrolyzed to lysine and other products in strong acid. The amount of lysine appearing on the column thus depends upon the duration of hydrolysis in 6 N HCl at 110°. The rate constant for ε-acetimidolysine hydrolysis was determined by observing the rate of increase of lysine from fully acetimidated RNase over a period of 72 hr. A pseudo-first-order reaction was observed. The rate constant is 1.5×10^{-2} hr^{-1} and the half-life, 46 hr. Therefore, the amount of substituted lysine present in any preparation of AIR can be determined from a simple first-order rate equation or graphically.

AIR has only 4% RNase activity toward RNA and is completely inactive toward uridine 2′3′-cyclic phosphate. It is apparently homogeneous from the ultracentrifuge data. Only one band appears on polyacrylamide gels and only one component is eluted from a SE-Sephadex column using 0.12 M sodium phosphate buffer (pH 6.5).

The rate of deacetimidation was studied at two different temperatures to determine which would be more advantageous in regenerating an active enzyme. The results of kinetic studies at 4 and 25° indicate the yield of active enzyme to be the same and since the reaction is about ten times faster at 25° all preparative deacetimidations were carried out at this temperature. As a control RNase was dissolved in the deacetimidating solutions at 25° and aliquots were assayed at various times to determine the amount of denaturation during ammonolysis of the acetimido groups. The inactive material precipitated during the reaction but was soluble in the assay solution (pH 5). If an aliquot containing the precipitate was assayed 2% activity was lost in 3 hr and 15% in 24 hr. In every case, if the precipitate were centrifuged before assay the supernatant contained 100% active RNase.

TABLE II: Physical Constants for RNase and AIR.

	RNase	AIR
$\epsilon_{2775\ Å}$	9800[a]	9900
$[\alpha]_{min}$	−4400 (2310 Å)[b]	−4100 (2250 Å)
$s^0_{20,w}$ (S)	1.8–2.2[a]	2.3
Tyr pK_{int}	9.9[c]	10.0

[a] Scheraga and Rupley (1962). [b] Glazer and Simmons (1965). [c] Tanford *et al.* (1955).

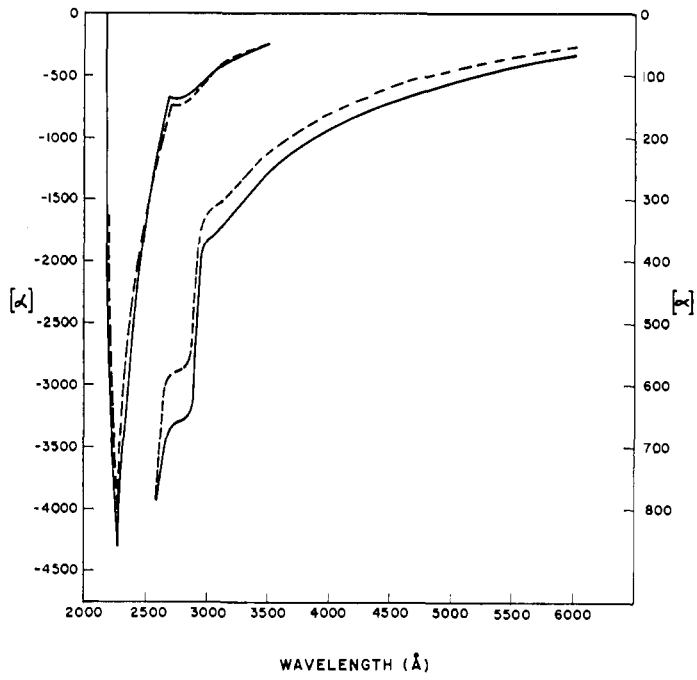


FIGURE 2: Optical rotatory dispersion spectra of RNase (———) and AIR (– – – –). Right ordinate 2500–6000 Å; left ordinate 2000–3500 Å.

A pseudo-first-order reaction was observed for the ammonolysis of AIR at 25°. The half-life for deacetimidation at this temperature is about 0.8 hr, the complete reaction requiring about 6 hr at 25° and about 2 days at 4°. The yield of active soluble products is approximately the same at both temperatures indicating the rate constant of inactivation has about the same temperature coefficient as the rate constant of deacetimidation. At both temperatures 100% activity is regained prior to full unblocking of the lysine (Figure 1). Examination of the soluble product of deacetimidation at 25° on SE-Sephadex shows that it is not identical with RNase A but elutes earlier from the column. Glycination showed that no new carboxylate groups had been formed.

Only three of the tyrosines in AIR can be titrated in 0.15 M KCl between pH 10 and 12 and their pK_{int} is 10.0 mimicking RNase behavior (Tanford *et al.*, 1955). The remainder of the measurements of physical constants of AIR and RNase are given in Table II and the optical rotatory dispersion spectra are compared in Figure 2.

Solutions of RNase and AIR which had been sub-

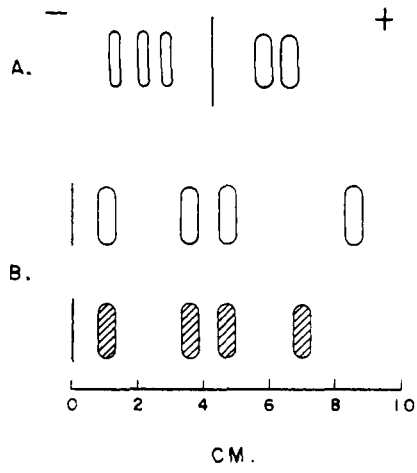


FIGURE 3: Tryptic peptides of AIROX. (A) Separated by cellulose acetate electrophoresis. (B) Separated by paper chromatography. Open areas represent staining with ninhydrin, the shaded areas with Sakaguchi stain.

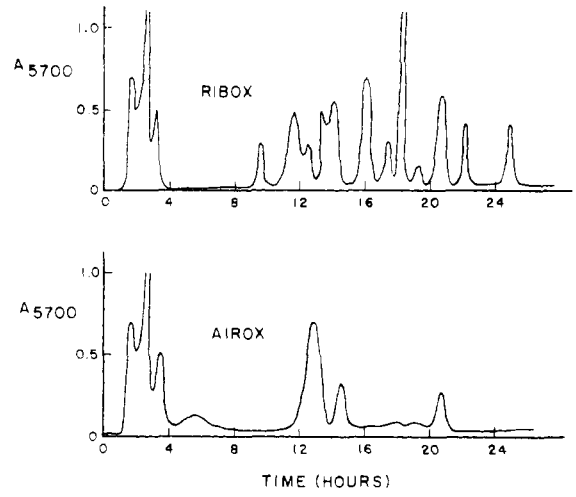


FIGURE 4: Comparison of ion-exchange chromatograms of tryptic peptides of RIBOX and AIROX.

jected to trypsin were analyzed by peptide mapping. No new ninhydrin- or Sakaguchi-positive areas occurred in the AIR digest when compared with controls of AIR and RNase, indicating that no hydrolysis had taken place. The tryptic digest of AIROX was analyzed by cellulose acetate electrophoresis and paper chromatography. The results are shown in Figure 3. On electrophoresis the five expected peptides are separated. Two move toward the anode and three toward the cathode. The same result was observed from peptides obtained by tryptic digestion of trifluoroacetylated performic acid oxidized RNase (Goldberger and Anfinsen, 1962). On paper chromatography the five peptides are also separated; four are ninhydrin positive and four Sakaguchi positive, since the N-terminal peptide contains no amino groups and the C-terminal peptide no arginine. The number of peptides obtained was compared with that of a trypsin digest of RIBOX by separation on a sulfonated polystyrene ion-exchange resin. Figure 4 shows the two chromatograms of the effluent from the column after basic hydrolysis and reaction with ninhydrin. The number of peptides has been reduced by an amount approximately equal to the number of lysines (10) in RNase.

Discussion

Methyl acetimidate has been shown to be a specific reagent for the amino groups of lysine and the α-amino groups in various model compounds and proteins (Hunter and Ludwig, 1962). This specificity apparently holds for RNase as well. Essentially all of the amino groups have been shown to be substituted with the acetimido group by three different techniques. The use of TNBS to determine the number of amino groups present in a protein is faster and simpler than the DNP method used previously for acetimido proteins (Wofsy and Singer, 1963) which requires the use of unusual buffers (Nishikawa *et al.*, 1967) on the amino acid analyzer for good ϵ-DNP-lysine determinations. However, the TNBS method suffers from the apparent difference in extinction coefficients of some of the ϵ-TNP-lysines and α-TNP-amino acids in some proteins (Habeeb, 1965). TNP-RNase has a higher extinction coefficient (1.5×10^5) than can be calculated from ten ϵ-amino groups (1.15×10^4) and one α-amino group ($1\text{–}1.25 \times 10^4$). The method of measuring the appearance of lysine during acid hydrolysis was especially useful during the deacetimidation studies since it was difficult to rapidly eliminate all ammonia from the deacetimidated protein.

RNase was found to slowly denature under the highly basic deacetimidation conditions. Fortunately this reaction was not injurious to deacetimidation of AIR since hydrolysis of the acetimido group is so much faster than denaturation and the inactive product is easily removed by centrifugation. Since the amino acid analyses of the products of deacetimidation of AIR show no change in amino acid composition and since the chromatographic difference is apparently not the result of hydrolysis of certain glutamines and/or asparagines the difference between the regenerated enzyme and RNase A remains unknown. Further work is being carried out to determine this difference.

Consistent with the properties of acetimidated bovine serum albumin, various rabbit antibodies (Wofsy and Singer, 1963), and insulin (Hunter and Ludwig, 1962) fully acetimidated RNase is very similar in its physical and chemical properties to its parent protein. This is a result which stems from the unhindered position of lysine residues in these proteins and the retention of a positive charge by the acetimidolysyl side chain. AIR differs from RNase in that it is enzymatically inactive and when oxidized is hydrolyzed by trypsin only at the arginine peptide bonds.[2]

Physical evidence for this similarity is summarized in

[2] Hunter and Ludwig (1962) found that an acetimidated RIBOX preparation (not AIROX in which the order of modifications are reversed) gave more than five peptides and proposed that some of the amino groups in RIBOX were not acetimidated as readily as others.

the constants listed in Table II. The sedimentation coefficient for AIR is that of a globular protein of the same size as RNase. The ultraviolet and optical rotatory dispersion spectra of AIR are nearly identical with that of RNase and three of the tyrosines remain untitrated at a pH of less than 12. These similarities suggest that the forces necessary for maintenance of the secondary and tertiary structure of RNase have not been changed upon acetimidation and AIR has the same spacial arrangements of the peptide backbone and amino acid side chains as RNase.

Even though AIR is enzymatically inactive to RNA and uridine cyclic phosphate it retains some of the other chemical properties of RNase. It is not susceptible to tryptic digestion at low temperatures and it will properly refold after its disulfide bonds have been reduced. This latter property shows that AIR like many other derivatives of RNase (Frensdorff *et al.*, 1967) retains the information in its primary structure to cause correct folding and the correct formation of the disulfide bonds.

In analogy with the guanidination of RNase (Klee and Richards, 1957) loss of enzymatic activity apparently arises from the substitution of lysine 41. This assumption follows from the work of Cooke *et al.* (1963) in which all of the lysines except lysine 41 in RNase could be polyalanated without loss of activity and from the carboxyalkalation studies of Heinrikson (1966).

Since acetimidation of proteins causes little or no changes in the secondary and tertiary structure it may be possible to use this property to detect the role that amino groups play in proteins in which it has been postulated, *e.g.*, bovine serum album in (Vijai and Foster, 1967), that they provide the counter ions for buried carboxylate groups. This work is now in progress.

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