proposals clearly expect an ordered mechanism when ammonia is used as well as when glutamine is used as substrate, neither is compatible with the random ATP–aspartate addition to the E–NH_2 complex that we found to occur.

The mechanism of the glutamine-dependent reaction proposed in this report (Figure 10) can be shown to be compatible with a random ammonia-dependent reaction. Figure 10 shows that glutamine and ATP bind before glutamate is released, and aspartate is then the last substrate to bind. Since asparagine synthetase has glutaminase activity, one can postulate that when ATP binds immediately after glutamine binds, it stabilizes the enzyme–ammonia complex, allowing the glutamate to be released without losing the ammonia. If no ATP were present, a glutaminase reaction would occur. The question as to why aspartate does not add before ATP is bound, during the glutamine-dependent asparagine synthetase reaction, has two possible explanations: (1) the presence of glutamate sterically prohibits aspartate from binding until glutamate is released, and (2) if ATP does not bind to the enzyme–glutamine complex, then the ammonia group is lost along with the glutamate. These conditions demand that the substrate addition be ordered when glutamine is the nitrogen source while a random addition can occur when ammonia is the nitrogen source for asparagine synthetase. Thus, both mechanisms proposed in this paper can be shown to be mechanistically compatible.

It is noteworthy that Milman et al. (1980) proposed the same order of release of PP_i, AMP, and asparagine as is proposed in this paper, even though different methods were used. Milman et al. (1980) observed the inhibition by all products with respect to each substrate. In the present study, the use of dual inhibitor studies and selected product inhibition studies greatly decreased the experimentation needed to arrive at the same conclusions. This shows the great potentiality of dual inhibitor studies where the presence of two or more inhibitors produces a multiplicative effect. It has not been cited extensively in the literature, but we feel it has potential for answering numerous important kinetic questions [see, for example, Krull & Schuster (1980)].

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Heterogeneity of Bovine Seminal Ribonuclease†

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ABSTRACT: Bovine seminal ribonuclease, a dimeric protein found to be homogeneous by several standard criteria of purity, is heterogeneous when analyzed by ion-exchange chromatography on (carboxymethyl)cellulose. Three increasingly cationic subforms can be separated. The heterogeneity is due to the presence of two types of subunits, α and β, which make up three isoenzymic dimers: α_2, β_2, and $\alpha\beta$. Deamidation reactions can convert the most cationic β_2 subform into the $\alpha\beta$ subform, which in turn can be converted into stable α_2 subform. These conversions involve the hydrolysis of 2 mol of differentially labile amide groups per mol of protein. The ratios α_2:$\alpha\beta$:β_2 are constant in all preparations of seminal ribonuclease tested; they are independent of the purification procedure as well as of the biological source of the enzyme (seminal plasma or seminal vesicles). These results indicate that deamidations occur in vivo before the protein is secreted from the seminal glands. They also suggest that heterogeneity of seminal ribonuclease reflects a physiological need of distinct molecular forms of enzyme or, alternatively, a process which leads to the aging of the protein.

Cases of exhaustively purified proteins, found to be heterogeneous even after they had been defined as homogeneous by standard criteria of purity, are not uncommon. In many cases, heterogeneity was found to be due to different degrees of glycosylation of a unique protein moiety; in other cases, it was evident that deamidation processes, often occurring as artifacts of purification, could generate multiformity in a protein preparation. The results we obtained studying the heterogeneity of bovine seminal ribonuclease provide satisfactory evidence both for deamidation as being the cause of heterogeneity and for its occurrence in vivo before isolation of the protein is undertaken.

Bovine seminal ribonuclease isolated from bull semen (D'Alessio et al., 1972a) or from seminal vescicles (De Prisco et al., 1972) had in fact been found to be homogeneous by several criteria of purity, including polyacrylamide gel electrophoresis, amino acid and ultracentrifuge analyses, and N- and C-terminal group determinations (D'Alessio et al.,

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1972a–c). The protein is dimeric (D'Alessio et al., 1972b), with two subunits linked by two interchain disulfide bridges as well as by noncovalent interactions (Di Donato & D'Alessio, 1973; D'Alessio et al., 1975), and a unique amino acid sequence has been determined for the subunit chain (Suzuki et al., 1976).

Here we wish to report how, under defined conditions of ion-exchange chromatography, the apparently homogeneous protein can be resolved into three components. The heterogeneity is found to be due to the presence in native preparations of bovine seminal RNase[1] of three isoenzymic forms of the enzyme, made up of two different types of subunits. The more cationic forms can be converted into the more anionic ones through deamidation reactions.

Furthermore, the results clearly indicate that these reactions and the resulting heterogeneity of seminal ribonuclease are due neither to artifacts of purification nor to any interactions of the protein with the ion-exchange resin.

Experimental Procedures

Materials. Seminal RNase[1] was purified as described (D'Alessio et al., 1972a) from bull seminal plasma, obtained from bull sperm after removal of spermatozoa by centrifugation. The sperm was kept frozen at −20 °C until the preparation of the enzyme was started.

Bull seminal vesicle secretion was obtained by manual expression from glands supplied by the slaughterhouse and kept at 0 °C for a few hours. The secretion, if not used immediately, was stored at −20 °C.

The monomeric, selectively S-carboxymethylated derivative of seminal RNase was prepared with iodo[^{14}C]acetic acid (Amersham, U.K.) as previously described (D'Alessio et al., 1975). All preparations, of native enzyme and of its derivative, were stored as lyophilized powders at −20 °C.

L-Glutamate dehydrogenase (type II), NADPH, yeast RNA (type II-RS), cytidine cyclic 2′,3′-phosphate, and ADP were Sigma products. *O*-(Carboxymethyl)cellulose (CM-32 grade) was supplied by Whatman.

Ammonia Estimation. Ammonia contamination in the incubation and the assay mixtures was minimized by the following procedure. All solutions were prepared with freshly twice-distilled water. Incubation mixtures, prepared by dissolving suitable amounts of lyophilized protein in ammonia-free buffers, were degassed and sealed under vacuum before incubation was started. Protein concentration was 2.2 mg/mL. For each determination, duplicate samples and protein-free blanks were incubated at 37 °C.

Ammonia content was estimated according to the method of Da Fonseca-Wollheim (1973) with the following modifications. Duplicate sample aliquots of 0.15 mL were withdrawn from the incubation mixtures at the appropriate time intervals and added to 0.85 mL of a premixed reaction mixture. This contained 0.1 M Tris-HCl, pH 8.6, 10 mM sodium α-ketoglutarate, 1 mM ADP, and 100 μM NADPH. A first absorbancy reading (A_0) at 340 nm was taken before the reaction was started by adding 1 μL of a solution of L-glutamate dehydrogenase (20 mg/mL). When the reaction end point was reached, the final absorbancy value (A_f) at 340 nm was recorded. The $\Delta A_{340} = A_0 - A_f$ was in a linear relationship with the ammonia content in the concentration range 3–30 μM. The method was calibrated with standard ammonia solutions (Preciset, supplied by Boehringer).

[1] Abbreviations used: RNase, ribonuclease; CM-cellulose, *O*-(carboxymethyl)cellulose; Tris, tris(hydroxymethyl)aminomethane.

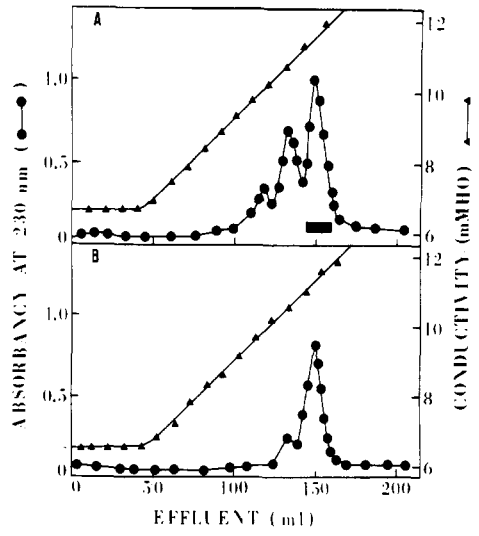

FIGURE 1: Salt gradient elutions from CM-cellulose (A) of bovine seminal ribonuclease, and (B) of the fractions corresponding to the main component, separated as shown in (A) and combined as indicated by the solid bar.

Other Methods. Assays for enzymatic activity on yeast RNA and cytidine cyclic 2′,3′-phosphate were performed as described before (Floridi et al., 1972). Amino acids and neutral and aminated sugars were analyzed by standard, previously described procedures (D'Alessio et al., 1972a). Proteins were concentrated from dilute solutions at 4 °C with immersible molecular separators (Millipore).

Results

Heterogeneity of Bovine Seminal Ribonuclease. The most convenient chromatographic system both for rapidity and effective resolution of seminal RNase subforms was a linear salt gradient on (carboxymethyl)cellulose columns (0.9 × 25 cm). The gradient was built with 100 mL of 0.1 M Tris-HCl at pH 8.4 containing 0.1 M NaCl in the mixing chamber and 100 mL of the same buffer, but containing 0.3 M NaCl, in the reservoir. The flow rate was 20 mL/h, and 2-mL fractions were collected. From 2 to 50 mg of protein could be analyzed in less than 10 h. A typical analysis is shown in Figure 1A. Seminal RNase, purified to homogeneity according to standard criteria of purity (D'Alessio et al., 1972a), was resolved, with the chromatographic system described above, into three components, which will be referred to as A subform, the most anionic species, an intermediate B subform, and C subform, the most cationic species. The ratios A/B/C, found to be constant for all the preparations tested so far, were equal to 1:3:6. Occasionally a minor inactive contaminant, which did not bind to the resin, was detected.

For investigation of whether the heterogeneity was due to interactions between the protein and the ion exchanger, the fractions corresponding to the C subform were combined as shown in Figure 1A and concentrated by ultrafiltration. After equilibration with the gradient starting buffer by gel filtration on a Sephadex G-25 column (1.5 × 15 cm), the material was rechromatographed with the gradient system described above. Figure 1B shows that upon rechromatography the C subform is eluted at an unchanged elution volume. The presence of a small amount of B subfform may likely be due to mere contamination in the combined fractions from the first chromatographic column.

The possibility was then investigated that heterogeneity reflected different amino acid compositions of the three subforms or different contents of carbohydrates. However, amino acid analyses of all three chromatographically pure components

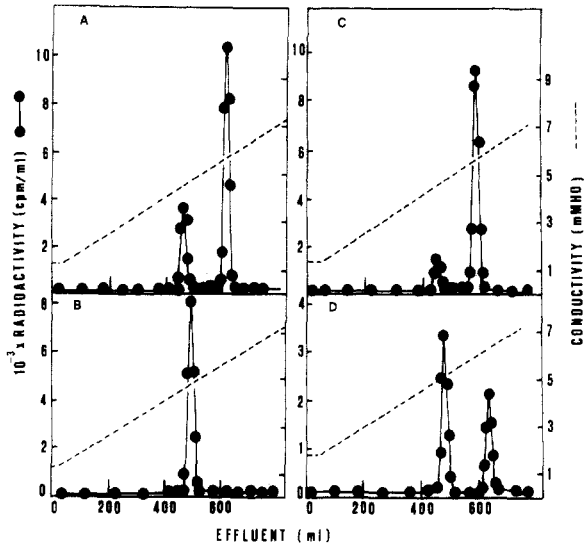

FIGURE 2: Salt gradient elutions from CM-cellulose of selectively reduced [^{14}C]carboxymethylated monomers prepared from (A) bovine seminal RNase, (B) A subform, (C) B subform, and (D) C subform.

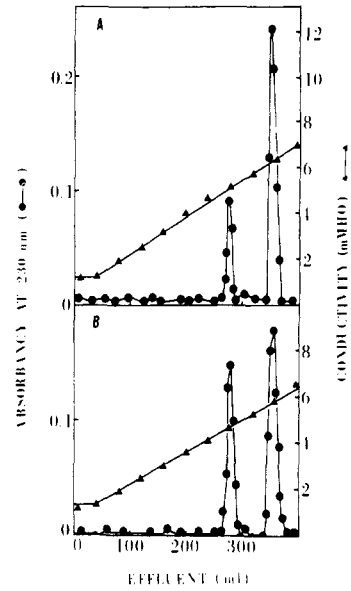

FIGURE 3: Salt gradient elutions from CM-cellulose of selectively reduced and carboxymethylated monomers of bovine seminal RNase (A) before incubation, and (B) after 50 h of incubation at 37 °C, in 0.04 M Tris-HCl, pH 7.8.

revealed no differences in their compositions. Furthermore, the three subforms were found to contain no neutral nor aminated sugars. It should be underlined that Asn-X-Thr/Ser sequences, which are considered to be the sites of N-glycosidation in proteins (Jackson & Hirs, 1970), do not occur in the amino acid sequence of seminal RNase (Suzuki et al., 1976; Beintema et al., 1976).

No differences in enzymatic activity were detected when the subforms of seminal RNase were tested with the standard assays on yeast RNA or on cytidine cyclic 2',3'-phosphate.

Minor differences in subunit structure were then considered as possible causes of heterogeneity. Bovine seminal RNase was thus dissociated into subunits and then subjected to ion-exchange chromatography. Dissociation was achieved by a previously described procedure (D'Alessio et al., 1975). This included (i) selective reduction of the intersubunit disulfides, followed by carboxymethylation of the exposed sulphydryls with ^{14}C-labeled iodoacetic acid, and (ii) mild denaturation with 3 M urea for disrupting noncovalent intersubunit interactions. The monomeric derivative produced by this procedure is stable, fully active (Parente et al., 1976) and has a conformation very similar to that of the homologous protein, pancreatic RNase A (Grandi et al., 1979).

Ion-exchange chromatography was carried out on a CM-cellulose column (0.9 × 25 cm) equilibrated in 0.04 M Tris-HCl, pH 7.8, and eluted with a linear gradient prepared with 400 mL of the equilibrium buffer in the mixing chamber and 400 mL of 0.1 M Tris-HCl, pH 8.4, containing 0.1 M NaCl in the reservoir (Goren & Barnard, 1970). A good resolution was obtained also with a gradient total volume of 400 mL.

When heterogeneous bovine seminal RNase, dissociated into monomeric subunits, was subjected to chromatography on the CM-cellulose column, two types of subunits were identified: α and β (Figure 2A). After a series of experiments, the ratio α/β between the more anionic component and the more cationic one was found to be equal to 1:2.9 (±0.3). Now a 1:3 ratio is exactly what was to be expected if the two types of monomeric subunits α and β were generated from the dissociation of three isoenzymic dimers α_2,$\alpha\beta$, and β_2 present in the ratios found for seminal RNAse subforms (1:3:6).

$$1\alpha_2 + 3\alpha\beta + 6\beta_2 \xrightarrow{\text{dissociation}} 5\alpha + 15\beta$$

This conclusion was further validated by preparing monomers from chromatographically homogeneous preparations of the three protein subforms. As it is shown in Figure 2, the A and C subforms gave rise to only one type of subunit. The monomer obtained from the A subform was eluted at an elution volume corresponding to that of α, the more anionic monomeric species present in native, heterogeneous seminal RNase, while the subunit type yielded from the C subform was identified with β, the more cationic species of monomeric seminal RNase. It should be noted that in contrast with the purity of the monomer obtained from the A subform, the β monomer prepared from the C subform was not homogeneous and appeared to be contaminated by a 12% of α. An explanation for this finding, consistently observed with several preparations, is presented below.

Finally, monomerized B subform was found to contain both the components α and β of the monomeric preparation of seminal RNase in a ratio of 1:0.76 (Figure 2D). Now, if a correction is made for a 12% contamination of α monomer in the β peak (the figure obtained in the experiments described above with homogeneous C subform), then a corrected ratio of 1:1, identical with the expected ratio, is obtained.

Deamidation Reactions Are the Basis of Seminal RNase Heterogeneity. The repeated observations, reported in the preceding paragraph, of the presence of contaminating α subunit in preparations of β subunit suggested the possibility that β subunit was not stable and could be transformed, under appropriate conditions, into α. Monomers of bovine seminal RNase were prepared as described in the preceding paragraph and then chromatographed on CM-cellulose before (Figure 3A) and after (Figure 3B) incubation in 0.04 M Tris-HCl, pH 7.8, for 50 h at 37 °C. Under these conditions, a substantial fraction of β subunit is transformed into α subunit, thus bringing the original ratio α/β of 1:2.9 to 1:1.4.

It was then conceivable that also β_2 and $\alpha\beta$ subforms were metastable and could be converted into $\alpha\beta$ and α_2 subforms, respectively. This possibility was investigated by incubating at 37 °C for different time intervals in 0.2 M potassium phosphate, pH 8.4, aliquots of a protein preparation obtained by chromatography on CM-cellulose of seminal RNase and consisting of about 80% of β_2 subform and 20% of $\alpha\beta$ subform (Figure 4A). After 19 h of incubation (Figure 4B), most of the β_2 subform was converted to the $\alpha\beta$ subform and sub-

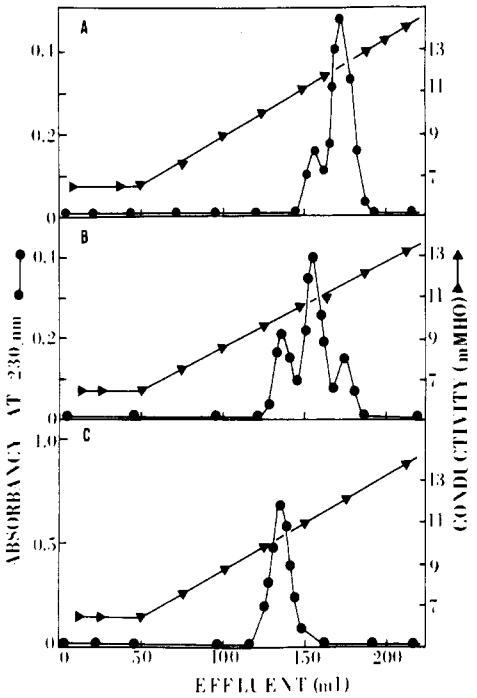

FIGURE 4: Salt gradient elutions from CM-cellulose of a preparation of β_2 subform of bovine seminal RNase containing 20% of $\alpha\beta$ subform (A) before incubation, (B) after incubation for 19 h at 37 °C, in 0.2 M potassium phosphate, pH 8.4, and (C) after 110 h of incubation under the same conditions.

stantial amounts of the $\alpha\beta$ subform into the α_2 subform. Upon a total of 110 h of incubation (Figure 4C), no more β_2 and $\alpha\beta$ subform could be detected, and all the protein was present as α_2 subform.

These results indicate not only that one subform can be easily converted into another but also that one subunit can be transformed into another. Furthermore, these conversions are unidirectional, i.e., from the more cationic into the more anionic species. In many experiments of incubation under several conditions of ionic strength, pH, and temperature (see below), no evidence was ever detected of a conversion from a more anionic subform or subunit into a more cationic one. Moreover, the results lead also to the conclusion that the α_2 subform appears to be a stable form of the protein.

Heterogeneity and the progressive increase with time of more negatively charged subforms of a protein can be interpreted in terms of amide hydrolysis of asparaginyl and/or glutaminyl residues of the protein. However, in order to prove that deamidation occurred, it is necessary to correlate the conversion of one protein form into a more anionic one with direct evidence of amide hydrolysis, such as ammonia release.

Ammonia determinations were carried out on the incubation mixtures analyzed by column chromatography in the experiments illustrated in Figure 4. Aliquots were withdrawn immediately before the samples were taken to the chromatographic columns and assayed for ammonia content as decribed under Experimental Procedures. It was found that 105 and 206 nmol of ammonia had been produced after 19 and 110 h of incubation, respectively.

For determination of the kinetics and the stoichiometry of ammonia liberation from the fully amidated subform, a sample of 80 nmol of chromatographically homogeneous β_2 subform was incubated at 37 °C in 0.2 M potassium phosphate, pH 8.4. Ammonia production was found to increase progressively in 120 h up to 156 nmol, corresponding to 1.95 mol/mol of dimeric protein, and then no further increase was detected up to 200 h of incubation. This finding and the results obtained in the experiments illustrated in Figure 4 indicate that under the conditions employed (i) a deamidation process is directly related to the conversion of one subform of seminal RNase into another, (ii) a stoichiometry of 2 mol of hydrolyzed amide per mol of dimeric protein can be calculated for the complete conversion of the β_2 subform into the α_2 subform, and (iii) the process goes to completion when all β_2 subform is transformed into α_2 subform and stops before any of the remaining 22 amides present in the molecule of seminal RNase are affected.

Effect of pH and Temperature on Deamidation of Seminal Ribonuclease. Conversions of β_2 subform into $\alpha\beta$ subform and of $\alpha\beta$ subform into α_2 subform were observed also at temperatures lower than 37 °C. After 76 h of incubation at 30 °C in 0.2 M potassium phosphate, pH 8.4, more than 30% of homogeneous β_2 subform was transformed into $\alpha\beta$ subform, while 5% of the protein was recovered as α_2 subform. At 4 °C, it was necessary to prolong the incubation for several days in order to detect any conversions. However, after 15 days of storage at this temperature, 50% of the β_2 subform was converted into the $\alpha\beta$ subform and 15% into the α_2 subform. The β_2 and $\alpha\beta$ subforms were not stable even when stored at −20 °C; after 15 days, about 30% of the original β_2 subform was recovered as $\alpha\beta$ subform, with traces of the α_2 subform.

Extent of deamidation as a function of pH was measured from the progress curves of ammonia released upon incubation at 37 °C in 0.2 M potassium phosphate (pH 8.4, 7.6, and 5.8) and in 0.2 M sodium acetate (pH 3.6 and 2.8). When the amount of ammonia released after 100 h of incubation of 2.2-mg aliquots of β_2-subform was plotted as a function of pH, a U-shaped curve, with a minimum at about pH 4, was obtained (data not shown). The curve is similar to the pH-dependence curves reported for hydrolysis of free amides or of protein amides (Flatmark, 1966; Robinson & Rudd, 1974).

These results offer also an explanation for the 12% contamination of subunit β in the monomeric preparation obtained dissociating subform β_2 (see Figure 2C and the corresponding paragraph). The time intervals and the conditions required by the procedures undertaken for the preparation of monomers, i.e., reduction and carboxymethylation (at pH 8.5) and chromatography (at pH 7.8–8.4), could easily generate a significant deamidation in a fraction of the fully amidated β subunit. In fact we found that only storage in the dry form preserved the purified protein from spontaneous deamidation. On the other hand, we also observed that no conversions of one subform into another ever occurred in crude preparations of the enzyme or when the enzyme was present in the original biological sources.

Heterogeneity of Seminal Ribonuclease Is Not an Artifact of Purification. The finding that both the presence of the subforms and their ratios were constant in all the preparations tested and, on the other hand, the interest to verify whether the partial deamidation generating heterogeneity of seminal RNase was an in vivo process led us to investigate if it could be excluded that the heterogeneity of seminal ribonuclease was due to deamidation occurring during the purification of the enzyme.

Seminal RNase was thus purified according to alternative purification procedures. One included an ammonium sulfate fractionation step followed by chromatography of the active fraction on phosphocellulose (Floridi & De Prisco, 1973). In another method (R. De Prisco, unpublished results), the cation exchanger was sulfopropyl-Sephadex C-50. The enzyme, purified with either procedures, was found to satisfy the criteria of purity applied to the enzyme preparations obtained with the standard procedure (D'Alessio et al., 1972a). Likewise,

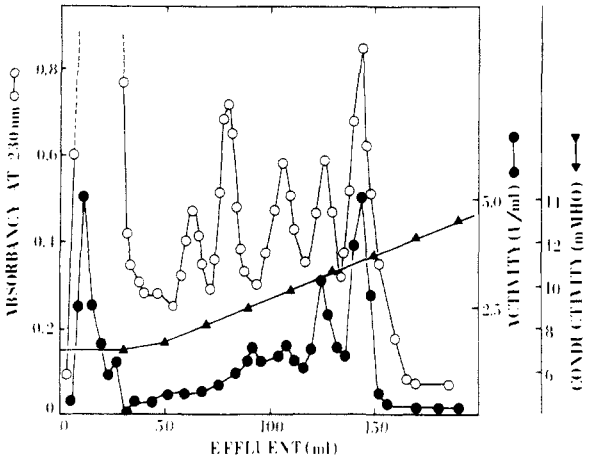

FIGURE 5: Salt gradient elution from CM-cellulose of a sample (2 mL) of bovine seminal plasma. Immediately before loading, the sample was diluted to a protein concentration of 20 mg/mL and adjusted to the pH and ionic strength of the chromatography starting buffer.

independently of the procedure employed for purification, all enzyme preparations gave an identical subform pattern with identical $\alpha_2/\alpha\beta/\beta_2$ ratios.

Bovine seminal RNase can also be isolated from bull seminal vesicles (De Prisco et al., 1972), the organ which secretes most of the fluid part of semen (Mann, 1964). The procedure, which requires tissue homogenization in 0.75 N sulfuric acid, ammonium sulfate fractionation and ion-exchange chromatography, is different from the procedure used for purification of the enzyme from seminal plasma. We tested several preparations of enzyme purified from seminal vesicles and found that it displayed upon chromatography on CM-cellulose a subform pattern identical with that shown in Figure 1A for the enzyme prepared from seminal plasma. Moreover, the $\alpha_2/\alpha\beta/\beta_2$ ratios were also identical.

Finally, as all these purification procedures required several days and this could have accounted for at least some conversions of one subform into another, seminal plasma was tested for subforms by directly applying it onto the ion exchanger. A sample of 2 mL of plasma was brought to the pH and ionic strength of the chromatography starting buffer and then chromatographed on CM-cellulose as described for the purified enzyme. When the eluate was assayed for RNase activity, three activity peaks were identified as α_2, $\alpha\beta$, β_2 on the basis of their elution volumes (see Figure 5). Their ratios were virtually the same as those obtained with purified preparations of the enzyme. Activity was present also on the anionic side of α_2 and in the protein fraction which did not bind to the ion exchanger. This activity is to be identified with the minor RNase components of bovine seminal plasma (D'Alessio et al., 1981).

Discussion

The results presented above show that bovine seminal ribonuclease as purified is a heterogeneous protein. Three components of different net charge, A, B, and C subforms, can be separated by ion-exchange chromatography. The heterogeneity is not due to interactions of the protein with the ion exchanger nor to differences in amino acid compositions or sugar contents. It is based instead on different subunit compositions of the three subforms. Their dissociation yields two chromatographically distinct types of subunits, α and β, and their subunit structures can be defined as α_2, $\alpha\beta$, and β_2, respectively. The chemical basis of the difference between α and β subunits is their different amide contents. Upon incubation, irreversible transformation occurs of one subform, β_2 or $\alpha\beta$, into another with a more negative net charge, $\alpha\beta$ and β_2, respectively. When these transformations occur, ammonia is liberated. A study of the stoichiometry indicates that hydrolysis of two amide groups per dimeric molecule is required for a complete conversion of the most cationic subform into the most anionic one. The amide groups involved in deamidation are especially labile, as they are hydrolyzed even at −20 °C, while the remaining protein amides (22 per dimeric molecule) resist hydrolysis even at a temperature of 37 °C.

An important question then arises as to whether or not the two ammonia equivalents liberated per dimeric molecule are cleaved off a unique amide residue on each subunit chain. The generation of α and β subunits in a 1:1 ratio from dissociated $\alpha\beta$ subform and the production of a single homogeneous protein subform (α_2) from the deamidation of $\alpha\beta$-subform suggest that a unique differentially labile amide is present on the polypeptide chain of β subunit. Manjula et al. (1976) found that upon incubation of bovine pancreatic RNase in highly acidic media, a monodeamidated derivative of the protein is produced. Fingerprint analysis of the derivative indicated that the region of the polypeptide chain where the labile amide group is located was to be identified with the tryptic peptide corresponding to residues 67–85.

It seems clear that deamidation of labile amide group(s) of bovine seminal RNase occurs in vivo before the protein is released in seminal plasma. This view is supported by the following facts. The subform pattern and the ratios of one subform to the others were constant in all preparations tested. Furthermore, they were independent of the purification procedure employed and of the biological source of the enzyme. Finally, freshly collected bull semen was found to contain seminal RNase subforms in the same ratios as the purified enzyme.

Once the in vivo occurrence of the deamidation processes causing heterogeneity of seminal RNase has been established, it would remain to define the mechanism underlying such phenomenon and its biological role. In vivo deamidation of proteins has been previously reported (Flatmark & Sletten, 1968; Midelfort & Mehler, 1972). Robinson & Rudd (1974) also proposed that controlled deamidation of proteins has a role as "a general molecular timer of biological processes"; successive deamidations of a protein would lead to production of subspecies more susceptible than the original protein to proteolytic degradation.

Native heterogeneous seminal RNase, although found to be resistant to proteolytic degradation by trypsin, chymotrypsin, and subtilisin (Parente et al., 1976), could well be susceptible, in its partially deamidated form, to specific proteases of the seminal vesicle tissue.

An alternative hypothesis, of deamidation as a process leading to isozymes with different functional properties, would appear to be opposed by the finding that no differences in specific activity could be detected in the three enzyme subforms. However, this should be confirmed with a more thorough analysis of the catalytic properties of the enzyme subforms, carried out with a wider range of conditions and substrates. Furthermore, it should be stressed that, to be conclusive, such analysis should include the natural substrate(s) of the enzyme, which are as yet to be discovered.

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Activation and Chromatographic Properties of the AtT-20 Mouse Pituitary Tumor Cell Line Glucocorticoid Receptor†

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ABSTRACT: The physicochemical properties of the glucocorticoid receptor (GC-R) from the mouse AtT-20 pituitary tumor cell line were studied. Analyses involved ion-exchange (diethylaminoethylcellulose, phosphocellulose), gel filtration (Sephadex G-150), and adsorption (DNA–cellulose, hydroxylapatite) chromatography. The receptor was characterized in four different states: native (unactivated), activated, nuclear, and Na_2MoO_4 stabilized. The unactivated receptor had chromatographic properties which were very similar to those of other steroid hormone receptor proteins; that is, it was not adsorbed to phosphocellulose (PC) or DNA–cellulose but did adsorb to diethylaminoethylcellulose (DEAE-cellulose) and eluted at 0.2 M KCl. In addition, a single peak, which eluted at 0.11 M phosphate, was obtained upon hydroxylapatite (HAP) chromatography. Because of aggregation, it was not possible to estimate the size of the unactivated GC-R. After activation by Sephadex G-25 gel filtration or by precipitation at 40% saturated $(NH_4)_2SO_4$, the receptor adsorbed to both PC and DNA–cellulose, eluting in a single, symmetrical peak at 0.17 and 0.14 M KCl, respectively. The fact that the activated receptor was a more basic molecule was confirmed by DEAE-cellulose chromatography; the activated GC-R did adsorb but eluted earlier as a sharp peak at 0.08 M KCl. The physicochemical properties of the receptor which are responsible for adsorption to HAP were unchanged after activation, since the activated GC-R eluted identically with the unactivated GC-R at 0.11 M phosphate. Sephadex G-150 gel filtration of the activated GC-R yielded a Stokes radius of 6.8 nm. The characteristics of the nuclear receptor were identical with those of the in vitro activated cytosol receptor. Finally, Na_2MoO_4 appeared to stabilize the unactivated form of the receptor. The Stokes radius of the MoO_4^{2-}-stabilized receptor was 7.7 nm. No heterogeneous forms were detected after activation on any chromatographic system utilized. That is, there was no evidence of dissimilar steroid-binding subunits. Also, limited proteolysis of the glucocorticoid receptor was not seen in this cell line. Thus, it is most likely that activation of the glucocorticoid receptor in this mouse pituitary tumor cell line is due to a conformational change in the protein or the dissociation of identical subunits.

All steroid hormones are now believed to act via a similar scheme (Yamamoto & Alberts, 1976). This involves the uptake of the hormone into the cell followed by a specific, saturable, noncovalent binding of the hormone to an intracellular, soluble receptor protein. Subsequent to ligand binding, the receptor undergoes a poorly understood process called "activation" [sometimes referred to as "transformation" (Sando et al., 1979)]. This results in the accumulation of the hormone–receptor complex in the nucleus, which is believed to be its primary site of action. It is not yet clear whether nuclear translocation involves the acquisition of the ability to penetrate the nuclear membrane or is due to a dramatic switch

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