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Articles

Selective Deamidation and Enzymatic Methylation of Seminal Ribonuclease[†]

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Received July 29, 1986

ABSTRACT: Isoenzymatic forms α_2, $\alpha\beta$, and β_2 of bovine seminal ribonuclease are generated by the transformation of β-type into α-type subunit through deamidation of a single amide group [Di Donato, A., & D'Alessio, G. (1981) *Biochemistry 20*, 7232-7237]. The residue involved in this selective deamidation has been identified as Asn^{67}. Deamidation occurs by formation of a cyclic imide intermediate involving the Gly at position 68. Opening of the cyclic imide may occur on either side of the nitrogen, generating both the normal α-aspartyl and an isoaspartyl residue at position 67. The α-carboxyl of the isoaspartyl residue is effectively methylated by bovine brain protein carboxylmethyltransferase.

Bovine seminal ribonuclease (BS-RNase)[1] is a ribonuclease isolated from bovine seminal plasma and seminal vesicles (D'Alessio et al., 1972; Tamburrini et al., 1986). It is a dimer, with two disulfides and noncovalent forces linking together the two subunits (Di Donato & D'Alessio, 1973; D'Alessio et al., 1975). In an earlier paper (Di Donato & D'Alessio, 1981) it has been reported that (1) the enzyme, as isolated, is a mixture of three isoenzymic forms, α_2, $\alpha\beta$, and β_2, (2) β-subunit transforms into α-subunit upon hydrolysis of a single amide group, and (3) the production of isoenzymes has to be physiological, as the isoenzymes are consistently found, and in the same ratios, irrespective of the enzyme source (seminal plasma or seminal vesicle tissue) and of the isolation procedures. Two main questions were left unsolved: the identification of the selectively labile amide residue and the mechanism of deamidation.

We report here on the identification of the asparaginyl amide group, in the Asn^{67}-Gly^{68} sequence, as the amide selectively hydrolyzed when β-subunit transforms into α-subunit and on the mechanism of deamidation, involving a cyclic imide intermediate, which spontaneously hydrolyzes, producing either a normal aspartyl or an isoaspartyl residue. The finding that the free α-COOH of the latter can be stoichiometrically methylated by the enzyme *S*-adenosylmethionine:protein carboxyl-*O*-methyltransferase (protein methylase II) provides a further example, after that of porcine adrenocorticotropin (Aswad, 1984; Murray & Clarke, 1984), on the involvement of deamidation at Asn-Gly sequences in the generation of methylatable sites in proteins. It may also provide a basis for an understanding of the possible physiological meaning of the selective deamidation leading to the transformation of β- into α-subunit of BS-RNase. This in turn may be used as a suitable experimental system for studying the general significance of the production of methylatable α-COOH groups in proteins.

EXPERIMENTAL PROCEDURES

Selective Deamidation of BS-RNase. BS-RNase, purified from bull seminal plasma or from seminal vesicles (Tamburrini et al., 1986), was deamidated at 37 °C in 0.2 M potassium phosphate, pH 8.4, at a protein concentration of 2 mg/mL (Di Donato & D'Alessio, 1981). The extent of deamidation was monitored by fast-protein chromatography (Tamburrini et al., 1986) of aliquots of the deamidation mixture. Under these conditions, a virtually complete transformation of heterogeneous protein into a homogeneous preparation of selectivity deamidated subform α_2 was obtained. Purification of subforms was carried out by ion-exchange chromatography on (carboxymethyl)cellulose as described (Di Donato & D'Alessio, 1981).

Preparation of Fully Reduced and Carboxymethylated Proteins. Fully reduced and alkylated proteins were prepared

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[†] This work was partly financed by grants of the Italian Ministry of Education and the National Research Council, Italy.

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[1] Abbreviations: BS-RNase, bovine seminal ribonuclease; RNase A, bovine pancreatic ribonuclease A; ACTH, adrenocorticotropin; CM, carboxymethyl; RP-HPLC, reverse-phase high-pressure liquid chromatography; Na$_2$EDTA, disodium ethylenediaminetetraacetate.

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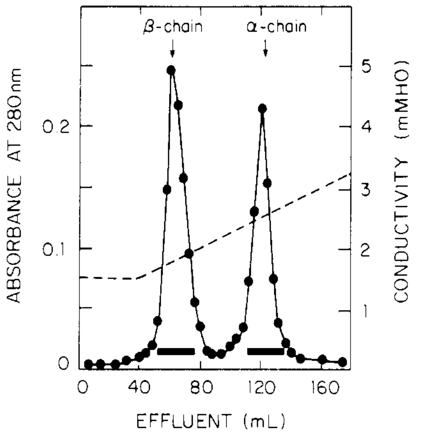

FIGURE 1: Separation on a DEAE-cellulose column of fully reduced and carboxymethylated α- and β-chains of BS-RNase selectively deamidated by incubation for 50 h at 37 °C in 0.2 M potassium phosphate at pH 8.4. The flow rate was 20 mL/min, and fractions were combined as indicated by the solid bars. Conductivity measurements (--) indicate the salt gradient used for elution.

at room temperature under a nitrogen barrier at a concentration of 5 mg/mL in 0.1 M Tris-HCl, pH 8.6, containing 6 M guanidine hydrochloride and 1 mM Na_2EDTA. Dithiothreitol was added to a molar excess over protein disulfides of 5:1. After 30 min, neutralized iodoacetic acid was added to a molar excess of 15:1 over protein disulfides. After 1 h in the dark, the protein was desalted on a Sephadex G-25 column eluted with 0.05 N acetic acid and lyophilized.

The procedure was applied either to homogeneous preparations of α_2 and β_2 subforms, purified as described (Di Donato & D'Alessio, 1981; Tamburrini et al., 1986), or to preparations of BS-RNase containing all three subforms (α_2, $\alpha\beta$, and β_2). In the latter case, after reduction and carboxymethylation, individual α- and β-chains were separated on a DEAE-cellulose column (Whatman DE-52, 0.9 × 25 cm) equilibrated with buffer A (0.04 M Tris-HCl at pH 8.4) and eluted by a linear salt gradient with 200 mL of buffer A in the mixing chamber and 200 mL of the same buffer containing 0.1 M NaCl (buffer B) in the reservoir. A typical separation is shown in Figure 1. Protein peaks were collected and immediately desalted by gel filtration on Sephadex G-25 columns eluted with 0.05 N acetic acid. Assignment of peaks was made by standardizing the DEAE-column with preparations of reduced and carboxymethylated α and β chains obtained from purified α_2 and β_2 subforms of BS-RNase.

Separation of Peptides. Peptides were separated by reverse-phase high-pressure liquid chromatography (RP-HPLC), with a Beckman instrument equipped with an Ultrasphere ODS column. Analytical runs were performed on a 5-μm column (0.46 × 25 cm), at a flow rate of 1 mL/min. When the material to be fractionated exceeded 100 nmol, a semipreparative 5-μm column (1 × 25 cm) was used at a flow rate of 3 mL/min. Peptides in the effluent were monitored by their absorbance at 210 nn, with a Knauer Model 87.00 UV detector. Elution of peptides was achieved with linear gradients of acetonitrile in 0.1% trifluoroacetic acid (Acharya et al., 1983). For an optimal separation of the tryptic peptides of α and β chains of BS-RNase (see Figure 2), the column was equilibrated and was loaded in 5% acetonitrile–0.1% trifluoroacetic acid; the concentration of acetonitrile was then raised to 12.6% in 23 min, kept constant for 5 min, raised again to 15% in 7 min, and then raised to 38% in 70 min.

Sequence Analysis. Edman degradation was carried out with 2.5% phenyl isothiocyanate (Pierce) in 50% pyridine on peptides (20–50 nmol) dissolved in 300 μL of the same solvent. The coupling step was carried out under nitrogen in screw-cap conical tubes for 45 min at 57 °C. Removal of excess phenyl isothiocyanate and of byproducts of the reaction was obtained by extraction with 3 times 0.5 mL of benzene (Beckman). The aqueous phase was taken to dryness under a stream of nitrogen, and the cleavage reaction was performed for 10 min at 57 °C with 100 μL of hydrofluorobenzoic acid (Beckman). The anilinothiazolinone derivatives were extracted from the samples with 3 times 0.5 ml of diethyl ether (Merck). The combined extracts, dried by centrifugation in vacuo in a Savant centrifuge, were incubated for the conversion to phenylthiohydantoins at 80 °C for 10 min in 50% trifluoroacetic acid. These were identified by HPLC as described (Pucci et al., 1983). Repetitive yields ranged from 78% to 81%.

COOH- and NH_2-Terminal Analyses. Samples were dissolved at a final concentration of 0.4 nmol/μL with norleucine as in internal standard for carboxypeptidase Y (Sigma) in 0.1 M sodium acetate, pH 5.6, and for aminopeptidase (Sigma type VI) in 1% ammonium bicarbonate. Each enzyme was added at an enzyme:peptide ratio of 1 μg/14 nmol, and incubations were carried out at 37 °C. Aliquots were withdrawn at the appropriate time intervals, brought to acidic pH by addition of 6 N HCl, heated at 100 °C for 2 min, and dried in vacuo in a Savant centrifuge. When the presence of glutamine was suspected in the sample, amino acid analysis was repeated with lithium buffers on a Beckman 119 CL instrument, following the procedure suggested by the manufacturer.

Determination of Isoaspartyl Residues. The presence of isoaspartyl residues was determined by the tritiation method devised by Matsuo et al. (1968) for the determination of COOH-terminal residues of peptides. Peptides (10 nmol) were dissolved in 200 μL of distilled pyridine and 100 μL of ^3H_2O (sp act. 5 Ci/mL, Amersham, England). Freshly distilled acetic anhydride (50 μL) was then added, and the reaction mixture was allowed to stand for 1 h at 0 °C and overnight at room temperature. After evaporation in vacuo, the residue was repeatedly washed with distilled water and subjected to hydrolysis in 6 N HCl. Identification of labeled amino acids was carried out on a Beckman 119 CL amino acid analyzer, collecting directly in scintillation vials 0.6-mL fractions of the eluate from the column of the analyzer. Counting was in Lumagel (Supelco, Italy). The amino acids eluting as radioactive peaks were identified on the basis of their elution volumes by comparison with a calibration run carried out with a ^{14}C-labeled amino acid mixture (ICN).

Hydroxylamine Cleavage. Cleavage with hydroxylamine was performed as described by Bornstein and Balian (1977) with minor modifications. Briefly, 1 mg of protein was dissolved in 0.9 mL of water at 0 °C in the vessel of a pH-stat apparatus. After addition of 1.72 g of guanidine hydrochloride and of 416 mg of hydroxylamine hydrochloride, the pH was brought to 8.6 with aliquots of a cold, saturated LiOH solution and the volume adjusted to 3 mL with chilled water. The reaction vessel was then covered with Parafilm and left for 2 h at 45 °C. The sample was then chromatographed on a Sephadex G-50 column eluted with 0.05 M ammonium bicarbonate.

Methyl Esterification of Peptides. Protein methylase II was purified 3000-fold from fresh bovine brain as described by Kim et al. (1978) by affinity chromatography on *S*-adenosyl-L-homocysteine–agarose (BRL), followed by gel filtration on Sephadex G-100. The purified enzyme preparation had a specific activity of 7000–9000 units/mg at 37 °C, with 20 μM deamidated porcine corticotropin (ACTH grade V, Sigma) as the methyl-accepting substrate. The protein was deamidated by treatment in 0.1 M ammonium acetate for 3 h at 37 °C and purified by (carboxymethyl)cellulose chromatography, as described by Aswad (1984).

The methyl esterification reaction was performed in 2.2-mL Eppendorf micro test tubes. The incubation mixture contained, in a final volume of 60 μL, 6 μL of citrate/phosphate/EDTA buffer, pH 6.2 (Kim & Paik, 1970), 4 μg of purified to homogeneity protein methylase II, 5 nmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine (Amersham, sp act. 100–300 dpm/pmol), and appropriate amounts of the peptides.

The reaction was initiated by adding the labeled substrate in a volume of 12 μL, and the incubation was carried out at 37 °C. The incorporation of [^{14}C]methyl into the peptides was evaluated by extracting with isoamyl alcohol the methanol released by alkali treatment (Jamalludin et al., 1976; Oliva et al., 1980). The reaction was stopped by addition of 60 μL of 0.5 M borate buffer at pH 10. After 1 min at 37 °C, isoamyl alcohol (1.25 mL) was added, and the tube was mixed vigorously for 15 s on a Vortex mixer. The layers were separated by centrifugation in an Eppendorf centrifuge for 3 min; an aliquot of 0.5 mL of the organic phase was mixed with 4 mL of scintillation fluid (Atom-light, New England Nuclear, West Germany) and counted for radioactivity.

Units of enzymatic activity are defined as picomoles of methyl groups transferred per minute per milligram of protein.

Other Methods. Trypsin (Sigma type XIII) was used with 1 mg/mL solutions of protein in 1% ammonium bicarbonate. Amino acid analyses were performed as described (D'Alessio et al., 1972). The autoanalyzers were a Beckman 119 CL and an LKB 4400.

RESULTS

Comparative Fingerprint Analysis of α- and β-Chain of BS-RNase. Previous results have shown that α-type subunit is transformed into β-type with the concomitant release of 1 mol of NH_3/mol of subunit (Di Donato & D'Alessio, 1981). In order to identify the amide residue of the β-subunit involved in this selective deamidation, tryptic digests of fully reduced and carboxymethylated α- and β-chains of BS-RNase were prepared by reacting the proteins with trypsin for 1 h at a 1/100 (w/w) ratio of trypsin to protein; after 1 h at 37 °C, an equal amount of trypsin was added, and the incubation was continued for another hour. The two fingerprints obtained by HPLC are illustrated in Figure 2. They show that the digests from α- and β-chains are very similar and contain an equal number of peptides.

Peptides were numbered in both patterns according to their elution order and given the prefix α or β according to the subunit type from which they had been isolated. They were directly subjected to amino acid analysis but for the doublets α11/α12 and β11/β12, which were first resolved into their components by rechromatography on the HPLC column with a more shallow gradient.

The amino acid composition and the yields of the peptides obtained from α-chain are reported in Table I. Presumptive sequence assignments, based on the amino acid compositions, are also given in Table I. These compositions, which allowed for unique assignments when compared to the known sequence of BS-RNase (Suzuki et al., 1976), accounted for the entire sequence of the protein (see Figure 3). Furthermore, the comparison also shows that (1) some sequence segments are represented by two peptides, with identical compositions, such as peptides α14 and α15 for the sequence region 63–76 and peptides α16 and α17 for the region 62–76 and (2) some of the bonds involving lysyl and arginyl residues are only partially cleaved by trypsin under the conditions chosen for hydrolysis.

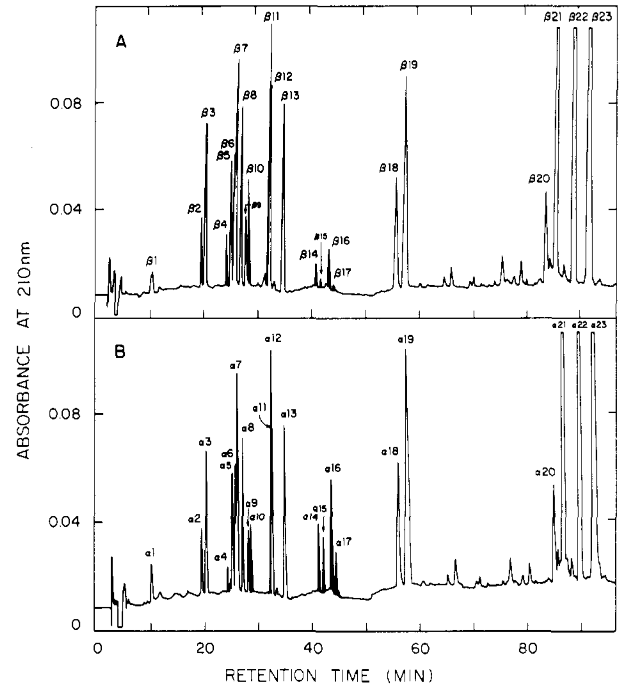

FIGURE 2: Separation by RP-HPLC of tryptic peptides of (A) β-subunit and (B) α-subunit chains of BS-RNase (5 nmol each). In black are the peaks where differences between the two patterns are detectable. Details of the gradient elution are given in the text.

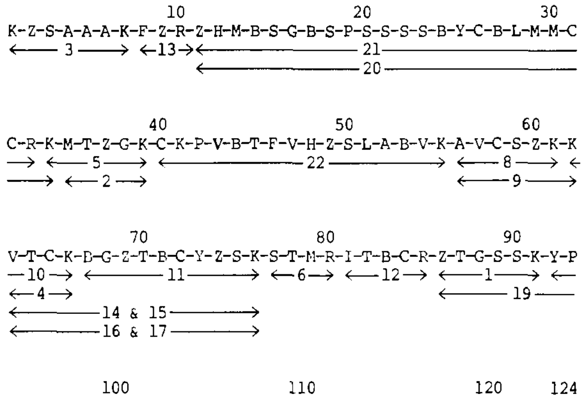

FIGURE 3: Tryptic peptides from α- and β-subunits of BS-RNase aligned on the amino acid sequence of the protein (Suzuki et al., 1976).

This occurred where basic residues were in pairs, as in the cases of Arg^{33}-Lys^{34} (cf. peptides α20 and α21 in Figure 3) and of Lys^{61}-Lys^{62} (peptides α8 and α9), and occurred also for the bonds Lys^{66}–Asx^{67} (peptides α14, α15, α16, and α17) and Lys^{91}–Tyr^{92} (peptide α19).

When the tryptic peptides from the β-chain digest were analyzed, their amino acid compositions were found to be identical with those of the corresponding peptides, with identical retention times, from the α-chain digest. Significant differences were instead found in the yields calculated for some of the β peptides. The couples of peptides with identical sequences (14/16 and 15/17), presumably with a Lys^{66}–Asx^{67} bond intact (see Figure 3), were obtained in very low yields (see Figure 2 and Table II). Conversely, the peptides generated by the cleavage of bond 66–67 were recovered in much higher yields than in the α digest (see Table II). Thus, it appeared as the peculiar events in tryptic hydrolysis were mainly a characteristic of the deamidated subunit.

The possibility that the conditions chosen for tryptic hydrolysis had affected the results was investigated by submitting

Table I: Amino Acid Composition of Tryptic Peptides from α-Subunit of BS-RNase[a]

	α1	α2	α3	α4	α5	α6	α7	α8	α9	α10	α11	α12
Asx											2.6 (2)	0.9 (1)
Thr	1.0 (1)	1.2 (1)		1.2 (1)	1.0 (1)	0.9 (1)	2.0 (2)			1.0 (1)	1.4 (1)	0.8 (1)
Ser	1.9 (2)	1.1 (1)	0.8 (1)			0.8 (1)		1.0 (1)	0.9 (1)		1.2 (1)	
Glx	1.2 (1)		1.1 (1)		1.1 (1)		2.4 (2)	1.1 (1)	1.1 (1)		2.1 (2)	
Pro												
Gly	1.3 (1)	1.0 (1)			1.0 (1)	0.3 (0)					1.3 (1)	
Ala			2.3 (3)					1.1 (1)	1.1 (1)			
Cys[b]				0.8 (1)				1.0 (1)	1.0 (1)	1.1 (1)	1.1 (1)	0.8 (1)
Val				1.1 (1)			1.0 (1)	0.5 (1)	1.3 (1)	1.8 (1)		
Met		1.0 (1)			1.0 (1)	0.8 (1)						
Ile												0.7 (1)
Leu												
Tyr											0.9 (1)	
Phe												
Lys	*1*	*1*	*2*	*1*	*2*		*1*	*1*	*2*	*2*	*1*	
His												
Arg						*1*						*1*
residue nos.	86–91	35–39	1–7	63–66	34–39	77–80	99–104	56–61	56–62	62–66	67–76	81–85

	α13	α14	α15	α16	α17	α18	α19	α20	α21	α22	α23
Asx		2.0 (2)	2.0 (2)	2.3 (2)	2.0 (2)	1.1 (1)	1.2 (1)	4.1 (4)	4.3 (4)	2.3 (2)	1.2 (1)
Thr		1.5 (2)	1.2 (2)	1.7 (2)	1.6 (2)		0.9 (1)			0.9 (1)	
Ser		0.9 (1)	0.7 (1)	1.0 (1)	1.0 (1)		1.9 (2)	5.4 (6)	5.1 (6)	1.2 (1)	2.0 (2)
Glx	1.2 (1)	2.0 (2)	2.1 (2)	2.4 (2)	2.4 (2)		1.1 (1)	1.0 (1)	1.1 (1)	1.0 (1)	
Pro						0.8 (1)	1.0 (1)	0.8 (1)	0.8 (1)	1.0 (1)	2.2 (2)
Gly		1.0 (1)	0.8 (1)	0.9 (1)	1.1 (1)		0.9 (1)	1.2 (1)	1.2 (1)		2.1 (2)
Ala						0.9 (1)	1.0 (1)			1.0 (1)	2.1 (2)
Cys[b]		2.1 (2)	1.8 (2)	2.5 (2)	2.2 (2)	1.0 (1)	0.9 (1)	2.3 (3)	2.3 (3)	1.3 (1)	0.9 (1)
Val		1.1 (1)	1.0 (1)	1.0 (1)	1.1 (1)					2.8 (3)	3.7 (4)
Met								2.6 (3)	1.0 (3)		
Ile											0.9 (2)
Leu								1.1 (1)	0.9 (1)	1.0 (1)	
Tyr		0.9 (1)	0.8 (1)	1.1 (1)	1.0 (1)	1.7 (2)	1.6 (2)	0.8 (1)	0.7 (1)		
Phe	1.0 (1)									0.9 (1)	1.0 (1)
Lys		*2*	*2*	*3*	*3*	*1*	*2*	0.8 (1)		*2*	*1*
His								1.1 (1)	0.9 (1)	0.8 (1)	1.5 (2)
Arg	*2*							*1*	*1*		
residue nos.	8–10	63–76	63–76	62–76	62–76	92–98	86–98	11–34	11–33	40–55	105–124

[a] Compositions are expressed as molar ratios relative to the number of Lys or Arg residues (italicized) per peptide molecule. Values in parentheses are the known compositions of the peptides (Suzuki et al., 1976). Amino acids contents lower than 0.3 mol/mol of peptide are not reported. [b] Measured as CM-Cys.

Table II: Yields of Tryptic Peptides from Sequence Region 62–76 of α- and β-Subunit of BS-RNase

peptide	yield (%)	peptide	yield (%)
α4	9	β4	27
α10	18	β10	55
α11	35	β11	78
α14	16	β14	<4
α15	11	β15	<2
α16	27	β16	<5
α17	9	β17	<1

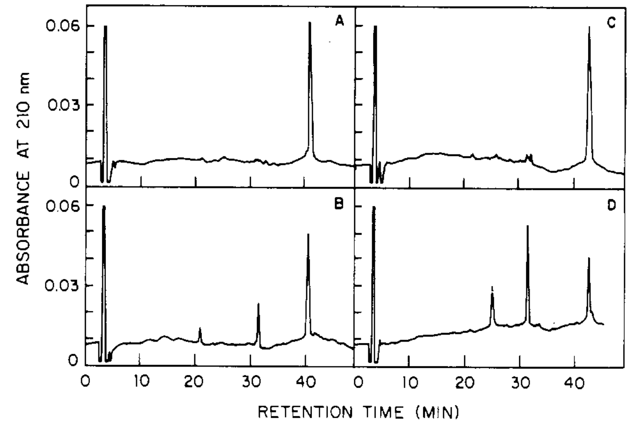

FIGURE 4: Effect of a further treatment with trypsin of tryptic peptides α15 and α17 from α-chain of BS-RNase. HPLC profiles of untreated peptides α15 (A) and α17 (C); HPLC profiles of digestion mixtures of peptides α15 (B) and α17 (D), after incubation with trypsin at a 1:50 molar ratio for 4 h at 37 °C. Elution gradients were as in Figure 2.

to a further treatment with trypsin the isolated peptides in which the bond Lys^{66}–Asx^{67} had not been cleaved, i.e., peptides α14, α15, α16, and α17. The results of this experiment indicated that, after 4 h of incubation with a 1:50 molar ratio of trypsin to peptide, peptides α14 and α16 were still found to be resistant to hydrolysis, whereas peptides α15 and α17 were cleaved to an extent of 30–50%, generating respectively peptides α4 and α11 and peptides α10 and α11 (see Figure 4). The peptides produced from hydrolysis were identified from their retention times upon HPLC and from their amino acid compositions.

These results were confirmed when reduced and carboxymethylated α-chain was submitted to a 20-h tryptic hydrolysis (data not shown). Peptides α15 and α17 were not found in the HPLC pattern of the hydrolysate, while the yields of peptides α4, α10, and α11 were much higher (by 50–75%) than those reported in Table II for a 2-h hydrolysis. The 20-h digest of β-chain was instead very much similar to the 2-h digest, except for the absence of peptide β19, which was cleaved into peptides β1 and β18.

These results indicate that in the subunit with a full complement of amide residues, i.e., β-subunit, trypsin readily cleaves the bond between the lysyl residue at position 66 and the residue following in sequence, whereas in the α-subunit,

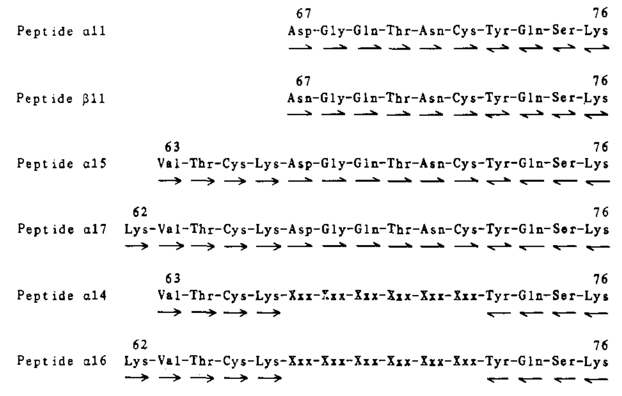

FIGURE 5: Sequence analysis of tryptic peptides from α- and β-chains of BS-RNase. Residues were identified by the Edman reaction (⇀) and/or by reaction with aminopeptidase (→) and carboxypeptidase (←) as described under Experimental Procedures. Residues at positions marked Xxx were not identified.

generated from β type by selective deamidation, this bond is either slowly hydrolyzed, as in peptides α15 and α17, or extremely resistant to hydrolysis, as in peptides α14 and α16.

Comparative Sequence Analysis of Region 62–76 from α- and β-Chains of BS-RNase. When the relevant peptides produced by tryptic cleavage from the sequence region 62–76 of α- and β-chains were submitted to sequence analyses, the results illustrated in Figure 5 were obtained: (1) at position 67 of β-subunit (in peptide β11) an Asn residue is located as in the amino acid sequence reported for the whole enzyme (Suzuki et al., 1976); (2) in the corresponding peptide α11 an Asp residue is found at position 67; (3) the sequences of peptides α15 and α17 are those presumed from their amino acid compositions (cf. Table I and Figure 3); (4) at position 67 the latter peptides have an Asp residue, instead of the Asn expected from the reported sequence of the protein (Suzuki et al., 1976); (5) peptides α14 and α16 could not be sequenced beyond the lysyl residue at position 66, after repeated trials with the Edman reaction and even prolonged incubations with aminopeptidase. On the basis of these data, a first conclusion can be reached that the selective deamidation transforming β-subunit into α-subunit takes place at Asn^{67}.

Mechanism of Selective Deamidation. The data as reported above, while defining the site of selective deamidation, left unresolved the question of the nature of peptides α14 and α16 and gave no clues as to the mechanism of deamidation. However, the presence of a Gly residue following in β-subunit the Asn at position 67 suggested that also in this case the mechanism of deamidation was based on a cyclic imide as an intermediate (see Figure 6). This mechanism, first proposed by Bornstein (1970), consists in a nucleophilic attack to the β-carbonyl group of the asparaginyl residue by the glycyl α-nitrogen. The resulting succinimide ring is spontaneously hydrolyzed on either side of the nitrogen, thus generating an isoaspartyl residue, linked by a β-peptide bond to the following Gly, besides regenerating the normal α-peptide bond.

We tested peptides α14 and α16 for the presence of an isoaspartyl residue at position 67 by reacting them with acetic anhydride in the presence of ^3H_2O according to Matsuo et al. (1968). It has been demonstrated that under these conditions only free α-COOH groups of the reacting peptides incorporate tritium through cyclization to oxazolones (Matsuo & Narita, 1975). It has been shown that also isoglutamyl residues react as they also possess free α-COOH groups (Matsuo et al., 1967). When α14 and α16 peptides were subjected to this reaction, they both were found, after hydrolysis and amino acid analysis, to contain radioactive lysine and aspartic acid (see Figure 7). While the finding of radioactivity in lysine was clearly due to its being the COOH-terminal residue in both peptides, radioactivity in the aspartic acid peak could only be reconciled with the presence of an isoaspartyl residue with a free α-COOH group in peptides α14 and α16. It has been shown (Manning, 1970) that β-hydrogen atoms of aspartic acid are exchanged with the medium during acid hydrolysis. This reaction could lead to aspecific incorporation of ^3H from residual ^3H_2O during hydrolysis. We thus carried out controls with peptides α15 and α17, with amino acid sequences identical with those of peptides α14 and α16, respectively. In the control peptides, only lysine was found to be labeled, ruling out incorporation of ^3H at the β-carbon of aspartic acid during acid hydrolysis.

The amino acid composition of peptides α14 and α16 indicate (cf. Table I) that two Asx are present in these peptides. By comparison with the amino acid sequences of the two related peptides α15 and α17, these Asx residues can be located at positions 67 and 71. The question of whether the

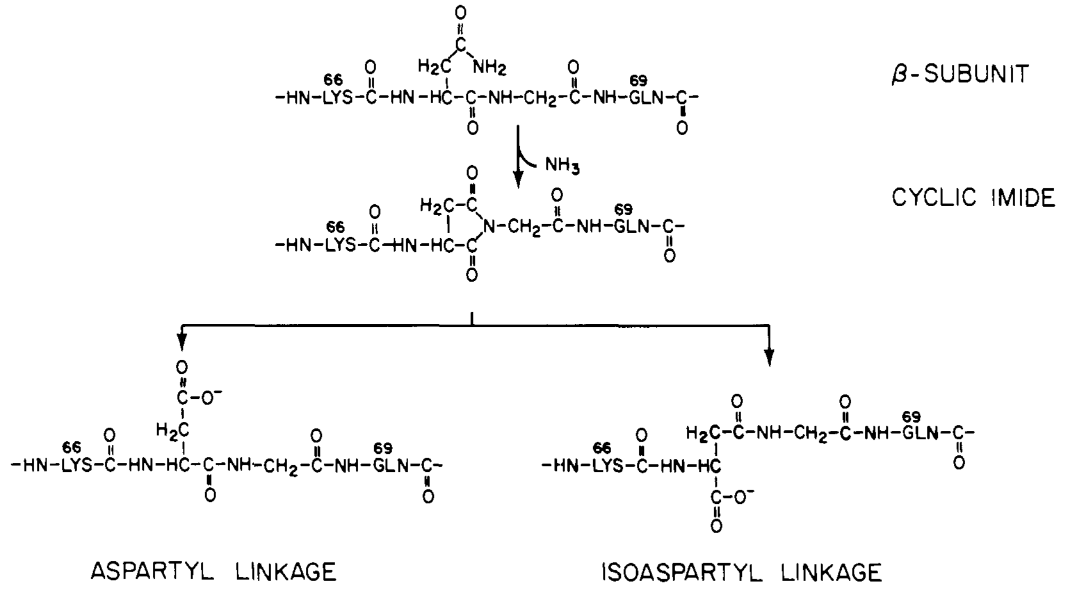

FIGURE 6: Proposed mechanism for deamidation of Asn^{67} in β-subunit of BS-RNase. The mechanism shown is based on that described by Bornstein and Balian (1977).

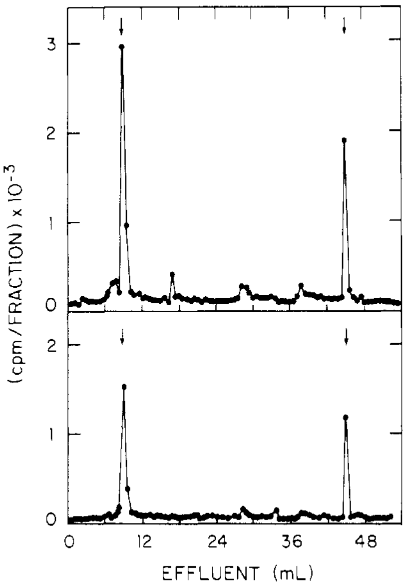

FIGURE 7: Radioactive amino acid analyses of peptides α14 (A) and α16 (B) labeled at their free α-COOH groups with ^3H_2O. Arrows indicate elution positions of [^{14}C]aspartic acid (9 mL) and [^{14}C]lysine (45 mL).

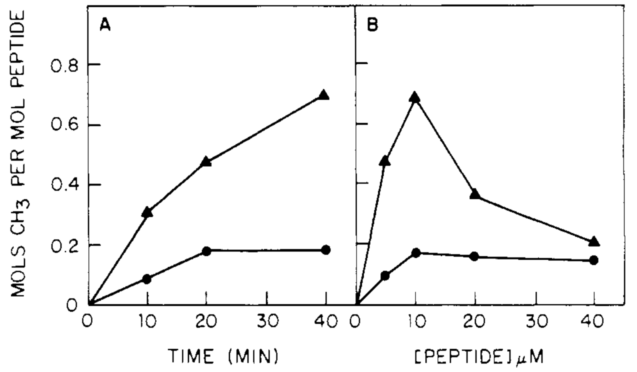

FIGURE 8: Enzymatic methyl esterification of tryptic peptides α14 (▲) and α15 (●) of BS-RNase as a function of time at a peptide concentration of 10 μM (A) and as a function of peptide concentration with an incubation time of 40 min (B).

isoaspartyl residue is at position 67 or 71 in these peptides could not be solved directly, for the lack of sequence data in the region of interest (see above). However, we can confidently locate the isoaspartyl residue of peptides α14 and α16 at their position 67, on the basis of the resistance of bond 66–67 of these peptides to trypsin and leucine aminopeptidase and of the resistance to cleavage by the Edman reaction of bond 67–68. It is well-known that (1) trypsin hydrolyzes very slowly peptide bonds adjacent to α-carboxyl groups (Neurath & Schwert, 1950; Waley & Watson, 1954), (2) leucine amino peptidase does not cleave isoaspartyl β-peptide bonds (Delange & Smith, 1971), and (3) cyclization to thioazolinone and cleavage do not occur in the Edman reaction when the NH_2-terminal residue is an isoaspartyl residue (Groskopf et al., 1966; Weber & Konisberg, 1967).

We also considered the possibility that the isoaspartyl residues found in the tryptic α-peptides were produced as artifacts during tryptic digestion and the subsequent experimental procedures, by hydrolysis of an imide ring initially present in the α-subunit. We thus treated with hydroxylamine (Bornstein & Balian, 1977) reduced and carboxymethylated α-subunit and found that it was not cleaved by the reagent, indicating that no imide ring was present.

Isoaspartyl Residues as Methylation Sites in BS-RNase. Recently, it has been shown that the α-carboxyl groups of isoaspartyl residues in corticotropin hormone (Aswad, 1984) and in a peptide from the same protein (Murray & Clarke, 1984) are good substrates for protein carboxylmethyltransferase, leading to stoichiometric methylation. We thus tested whether the α-COOH group of the isoaspartyl residues of the tryptic peptides of α-subunits of BS-RNase was a substrate for the enzyme purified from bovine brain. Figure 8A shows the kinetics of incorporation of methyl groups in peptide α14. Positive and negative controls were respectively deamidated porcine corticotropin (Aswad, 1984) and peptide α15, the latter presenting the same amino acid sequence as α14 but with an aspartyl residue at position 67 instead of an isoaspartyl residue. After 40 min of incubation, 0.7 mol/mol of peptide α14 was found to be methylated, while for peptide α15 only an initial incorporation was detected, likely due to contamination from the α14 peak in the HPLC separation (see Figure 2B). In order to characterize the ability of peptide α14 to act as a methyl acceptor in the reaction catalyzed by protein carboxylmethyltransferase, we studied the methylation reaction of peptide α14 as a function of peptide concentration. As is shown in Figure 8B, the optimal substrate concentration was found to be around 10 μM, possibly for the limited turnover of the enzyme during the finite incubation time of the assay (Aswad, 1984). As a similar value of optimum substrate concentration has been found also for deamidated corticotropin (Aswad, 1984) and for a synthetic peptide from this protein (Murray & Clarke, 1984), our finding indicates that isoaspartyl-containing peptide α14 is specifically recognized by protein carboxylmethyltransferase.

DISCUSSION

Bovine seminal ribonuclease is a heterogeneous dimeric protein whose subunits, α and β, differ for a single amide group and are related by a deamidation process that converts β- into α-subunits (Di Donato & D'Alessio, 1981). The results presented in this paper confirm that the only difference between the two types of subunits is an amide residue. This residue has been identified as Asx^{67}, which is amidated in β-subunit, whereas it occurs in deamidated form in α-subunit.

A surprising aspect of our results is that it was possible to locate the single residue variation between α- and β-subunit for the differential action of trypsin on the peptide bond linking Lys^{66} to the different residues following in sequence: an Asn residue in β-subunit, an Asp or an iso-Asp residue in α-subunit. In fact, we would have predicted that by classical fingerprinting analysis a difference of one peak would have arisen between the tryptic digests of the two subunits. Instead, under the conditions employed for peptide separation, i.e., an acetonitrile gradient in trifluoroacetic acid at pH 2, no differences in retention times between peptides α11 and β11 were detected, even with several acetonitrile gradients. Yet these peptides differ in their amino-terminal residue, which is Asn in β11 and Asp in α11.

On the other hand, the retarded action of trypsin on peptide bonds Lys^{66}–Asp^{67} and Lys^{66}–iso-Asp^{67} generates the only significant dissimilarity between the fingerprints of α- and β-subunit, i.e., the presence in the former of a quadruplet of peaks (peptides α14–α17), which is almost absent in the latter (see Figure 2).

This multiplicity of peptides cleaved off by trypsin from the same sequence region can be in part easily explained. Peptides α16 and α17 derive from a primary tryptic cleavage at the peptide bond linking lysyl residues 62 and 63 (See Figure 3). This cleavage produces peptides with NH_2-terminal lysine, which is known (Canfield, 1963) not to be further digested by trypsin. Peptides α14 and α15 derive instead from a tryptic cleavage between Lys^{63} and Val^{64}.

More peculiar appeared the finding that each type of cleavage generated two copies of the same peptide (α14/15 and α16/17). This was however clarified when the mechanism of deamidation, previously proposed in the literature (Bornstein & Balian, 1977) and illustrated in Figure 6, was considered and directly demonstrated by the presence in peptides α14 and α16 of isoaspartyl residues at position 67, whereas normal aspartyl residues were found at the same position in peptides α15 and α17.

To our knowledge, this is the first case that direct evidence has been obtained of the presence in a peptide chain of an iso-Asp residue generated through deamidation of an Asn residue. It was achieved through the judicious use of a procedure, the Matsuo reaction (Matsuo et al., 1969), originally developed for the determination of carboxyl-terminal residues in peptide chains. The present application, already foreseen by other authors (Matsuo et al., 1967; Cappugi et al., 1971), broadens the use of the reaction as a diagnostic tool for the occurrence of isoaspartyl residues in peptides and proteins.

The relative resistance of the peptide bond Lys^{66}–Asp^{67} in peptides α15 and α17 is explained considering that Lys^{66} is surrounded in these peptides by a cluster of negative charges, those of CM-Cys^{65} and of Asp^{67}. It is known that side chains with negative charges, adjacent to bonds potentially susceptible to tryptic cleavage, greatly reduce the rate of hydrolysis by trypsin (Kasper, 1975; Neurath & Schwert, 1950).

As for the extreme resistance of bond 66–67 in peptides α14 and α16, it can be reconciled with the presence at position 67 in these peptides of iso-Asp residues, which carry free α-COOH functions. It has been reported (Neurath & Schwert, 1950; Waley & Watson, 1954) that trypsin hydrolyzes very slowly peptide bonds adjacent to α-COOH groups, much more acidic ($pK = 2.0$) than β-COOH groups ($pK = 3.9$).

It is worthwhile to note that we have not detected an imide structure in the α-chain, as this was found to be resistant to hydroxylamine cleavage. On the other hand, an imide ring, as outlined in Figure 6, is a compulsory intermediate in the deamidation process leading to aspartyl and isoaspartyl bonds. Thus, resistance to hydroxylamine of the α-subunit chain, even after full reduction and S-carboxymethylation under denaturing conditions, suggests that formation of the imide ring is a very slow process in the deamidation reaction of BS-RNase, with a half-time of about 20 h (Di Donato & D'Alessio, 1981), whereas ring opening occurs at a high rate.

In the case of pancreatic RNase A, a protein strictly homologous to the subunits of BS-RNase (Suzuki et al., 1976), it has been demonstrated (Bornstein & Balian, 1970) that an imide ring is still present after, and possibly produced during, reduction and S-carboxymethylation under denaturing conditions. The imide ring is generated through deamidation of the Asn residue in the Asn^{67}–Gly^{68} sequence, which pancreatic RNase A shares with BS-RNase subunits. In fact, the protein chains of pancreatic and seminal RNases share the whole sequence between residues 65 and 75. This would imply that the rates of the individual reaction steps in the deamidation process depend not only on the primary structure of the protein but also on higher order structural elements.

The finding that the α-carboxyl groups generated after deamidation of Asn^{67} of BS-RNase are stoichiometrically methylated by carboxylmethyltransferase is intriguing. Methyl esterification of protein carboxyls in eucaryotic organisms has been extensively studied, but methylation has been consistently found to be largely substoichiometric (Kim & Li, 1979; Aswad & Deight, 1983; Barber & Clarke, 1983; Clarke & O'Connor, 1983). Recently, stoichiometric methyl transfer, catalyzed by protein carboxylmethyltransferase, was found to occur on the α-carboxyls of isoaspartyl residues generated through deamidation of the Asn residues in the Asn^{25}-Gly^{26} sequence of porcine corticotropin (Aswad, 1984) and of a synthetic peptide from this protein containing the same sequence (Murray & Clarke, 1984). A similar conclusion was arrived at on the basis of indirect evidence obtained with calmodulin (Johnson et al., 1985). These findings correlate at the molecular level the two processes of selective deamidation and methylation in proteins, a correlation already proposed and postulated to play a role in the processing of age-altered proteins (McFadden & Clarke, 1982; Galletti et al., 1983).

Although our data leave the latter question still open, they indirectly confirm that eucaryotic protein methylase II selectively recognizes proteins containing altered aspartyl sequences. It has been proposed (Aswad, 1984) that methylation at isoaspartyl residues generated through deamidation of Asn residues has a role in the repair of the protein chain altered by the presence of β-peptide bonds. However, in the case of BS-RNase, this hypothesis hardly fits with the available data. It has been reported (Di Donato & D'Alessio, 1981) that the enzyme is secreted as a mixture of isoenzymes, α_2, $\alpha\beta$, and β_2, in which the α-subunit represents about 25% of the total subunits; furthermore, the ratios among the three isoenzymes are constant; hence, constant is the α-subunit/β-subunit ratio. Here we report that α-subunit is constituted by more than 50% of peptide chains containing isoaspartyl residues at position 67 (see Table II and Figure 5). It seems unlikely that deamidation and the subsequent production of isoaspartyl residues at such an extent can be considered a fortuitous damage inflicted on the protein, to be repaired by methylation, followed by hydrolysis of the methyl ester The possibility can be surmised that a continuous recycling of peptide chains through steps of deamidation–methylation–demethylation–amidation is needed for maintaining the constancy of subunit ratio, hence a steady state of the levels of isoenzymic forms.

It should also be considered that the α_2 isoenzyme of BS-RNase is as active on RNA and on all the substrates tested as the β_2 isoenzyme (unpublished results), just like the α monomers are as active as the β monomers (Piccoli & D'Alessio, 1984). However, while the physiological role of the enzyme is unknown, BS-RNase has been found to exert, besides its enzymic action, also antitumor (Matousek, 1973; Vescia et al., 1980) and antispermatogenic action (Dostal & Matousek, 1973). The working hypothesis should be tested that deamidation and methylation of the generated isoaspartyls are molecular signals for other molecules or molecular structures, with which the protein has to interact for carrying out its yet unknown physiological action.

ACKNOWLEDGMENTS

We express our gratitude to Dr. Peter Blackburn for his crucial early suggestion on a possible mechanism of deamidation. We are grateful to Judith A. Gallea for her expert typing of the manuscript.

Registry No. RNase, 9001-99-4; L-Asp, 70-47-3; protein carboxymethyltransferase, 9055-09-8.

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Free Energy of Charges in Solvated Proteins: Microscopic Calculations Using a Reversible Charging Process[†]

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Received October 17, 1986

ABSTRACT: Evaluation of the free energy of ionization of acidic groups in proteins may be used as a powerful and general test case for determining the reliability of calculations of electrostatic energies in macromolecules. This work attacks this test case by using an adiabatic charging process that evaluates the *changes* in free energies associated with ionizing the acidic groups Asp-3 and Glu-7 in bovine pancreatic trypsin inhibitor and aspartic acid in solution. The results of these free energy calculations are very encouraging; the error range is about 1 kcal/mol for these free energy changes of about −70 kcal/mol. This indicates that we are finally approaching the stage of obtaining quantitative results in modeling the energetics of solvated proteins.

To correlate the structures of proteins with their functions, one must be able to express structures in terms of energies. Probably the most important requirement for such a correlation is the ability to evaluate the energies of charges in proteins. This is apparently the case in such key processes as proton transfer, electron transfer, subunit interaction, ion binding, and many enzymatic reactions. Thus, it is important to develop methods of calculation that yield reliable electrostatic energies in proteins (Warshel & Russell, 1984). In developing these methods it is essential to have experimental data capable of providing sufficiently discriminative test cases against which the calculations can be compared, so that incorrect or incomplete models may be discarded. Probably the

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[†] This work was supported by NSF Grant CHE-8519194, by NIH Grant GM-24492, and by the NSF San Diego supercomputer center.

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