Repair of Isopeptide Bonds by Protein Carboxyl O-Methyltransferase: Seminal Ribonuclease as a Model System†

P. Galletti,*,‡ A. Ciardiello,§ D. Ingrosso,‡ A. Di Donato,§ and G. D'Alessio§

Istituto di Biochimica delle Macromolecole, I Facoltà di Medicina e Chirurgia, Università di Napoli, 80138 Napoli, Italy, and Dipartimento di Chimica Organica e Biologica, Università di Napoli, 80134 Napoli, Italy

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ABSTRACT: Previous work has shown that in the peptide segment 62–76 of naturally deamidated α subunit of bovine seminal ribonuclease (BS-RNase) the α-carboxyl group of iso-Asp^{67} is selectively methylated by *S*-adenosylmethionine:protein carboxyl *O*-methyltransferase [Di Donato, A., Galletti, P., & D'Alessio, G. (1986) *Biochemistry 25*, 8361–8368]. In the present study this reaction has been characterized, by using the tryptic segment 62–76 of the protein chain (peptide α16). The peptide is stoichiometrically methyl esterified with a K_m of 6.17 μM and a V_{max} of 19.56 nmol min^{-1} mg^{-1}, and the product of demethylation has been identified as the cyclic succinimidyl derivative of iso-Asp^{67}-Gly^{68}. The cleavage of the succinimidyl ring yields two isomeric peptides containing an aspartyl residue (peptide α17) and an isoaspartyl residue (peptide α16). On the basis of these results conditions were defined in which repeated cycles of methylation–demethylation led to an effective conversion of peptide α16 into peptide α17, a process that can be interpreted as the repair of an altered isopeptide bond. When the methyl esterification reaction was studied on the native dimeric isoenzymes of seminal RNase and on catalytically active monomeric derivatives, including a stabilized α-type subunit, the results of these experiments showed that none of the protein forms were substrates for the methyltransferase. Only the unfolded α-type subunit was methylated to a stoichiometric extent. These results indicate that the repair of altered isopeptide bonds is chemically feasible in peptides but is hindered in the case of seminal RNase by its three-dimensional structure.

Stoichiometric methyl esterification of proteins and peptides, catalyzed by *S*-adenosylmethionine:protein carboxyl *O*-methyltransferase (PCMT;[1] EC 2.1.1.77), has been found to occur selectively at the α-carboxyl functions of L-isoaspartyl residues (Aswad, 1984; Clarke, 1985). Selective and stoichiometric methyl esterification was obtained by using proteins and peptides chemically or naturally deamidated at asparaginylglycyl sequences (Murray & Clarke, 1984; Johnson et al., 1985; Di Donato et al., 1986; Aswad et al., 1987).

Methyl esterification of the α-carboxyl function of isoaspartyl residues chemically introduced in Asp-X sequences of synthetic peptides has also been reported (Aswad & Johnson, 1987). However, it has been shown that deamidation of asparagine residues naturally produces isoaspartic residues, as it occurs via a cyclic succinimidyl intermediate, which by opening on either side of the imide nitrogen generates both a normal peptide bond and an atypical isopeptide bond, where the aspartyl is linked with its β-carbonyl to the glycyl following in the peptide chain (Bornstein & Balian, 1977; Di Donato et al., 1986). These altered aspartyl residues can occur in proteins as a consequence of age-dependent deamidation of specific Asn residues (Robinson & Rudd, 1974; Di Donato et al., 1986). The occurrence of an isopeptide bond as a consequence of Asp misincorporation during protein biosynthesis has also been suggested (Murray & Clarke, 1984).

Methylation of isoaspartic residues catalyzed by PCMT has been related to the repair of proteins containing isopeptide bonds (Johnson & Aswad, 1985; McFadden & Clarke, 1987; Johnson et al., 1987). The repair would occur through the following steps (see Figure 1): (1) enzymatic methylation of the α-carboxyls of iso-Asp residues; (2) cleavage of the methyl esters yielding succinimidyl residues; (3) hydrolysis of the cyclic succinimide and regeneration of the normal α-peptide bonds.

In order to test the validity of this repair strategy, we used as a model system bovine seminal ribonuclease (BS-RNase), a protein occurring in vivo in a partially deamidated form, and its relevant peptides, containing iso-Asp residues. BS-RNase, a dimeric protein, with two disulfides linking the two subunits (Di Donato & D'Alessio, 1973; D'Alessio et al., 1975), is a mixture of three isoenzymatic forms: α_2, $\alpha\beta$, and β_2 (Di Donato & D'Alessio, 1981). We have previously shown that α-type subunit of BS-RNase is generated through selective deamidation of β-type subunit at Asn^{67} and that an iso-Asp^{67} residue of a tryptic peptide of the α subunit is selectively and stoichiometrically methylated by PCMT (Di Donato et al., 1986).

In this paper we report the characterization of the methyl esterification reaction of the isoaspartyl residue of the α subunit of BS-RNase. This allowed us to define both the conditions for obtaining in vitro the repair of the isopeptide bond and the relationships between the structure of the protein and its competence as a methyl acceptor.

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* Author to whom correspondence should be addressed.

‡ Istituto di Biochimica delle Macromolecole, I Facoltà di Medicina e Chirurgia, Università di Napoli, Via Constantinopoli 16, 80138 Napoli, Italy.

§ Dipartimento di Chimica Organica e Biologica, Università di Napoli, Via Mezzocannone 16, 80134 Napoli, Italy.

[1] Abbreviations: BS-RNase, bovine seminal ribonuclease; RNase A, bovine pancreatic ribonuclease; M(*S*-CM)$_2$-BS-RNase, monomeric bis-(*S*-carboxymethyl)-Cys^{31},Cys^{32}-BS-RNase; M(SH)$_2$-BS-RNase, monomeric Cys^{31},Cys^{32}-BS-RNase; M(*S*-CM)$_{10}$-BS-RNase, fully reduced and carboxymethylated BS-RNase; M(SH)$_{10}$-BS-RNase, fully reduced BS-RNase; PCMT, protein carboxyl *O*-methyltransferase [*S*-adenosyl-L-methionine:protein-D-aspartate (L-isoaspartate) *O*-methyltransferase; EC 2.1.1.77]; AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosylhomocysteine; RP-HPLC, reverse-phase high-performance liquid chromatography; ACTH, adrenocorticotropin; EGF, epidermal growth factor; EDTA, ethylenediaminetetraacetic acid.

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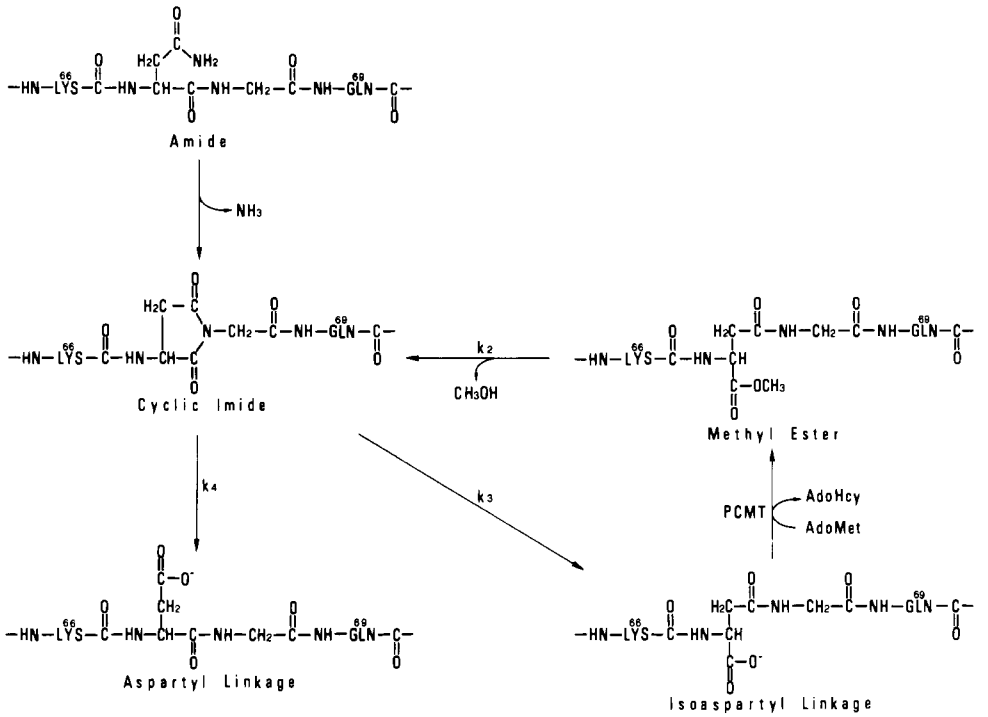

FIGURE 1: Proposed reaction scheme for deamidation of Asn[67] and repair of the resulting iso-Asp[67] of BS-RNase. For details, see text.

Experimental Procedures

Proteins. Purifications of PCMT, of BS-RNase, and of its isoenzymatic subforms were carried out as described (Di Donato et al., 1986). Adenosine deaminase from *Aspergillus oryzae* was purified as described (Zappia et al., 1974). Selective reduction of interchain disulfides and carboxymethylation of the exposed sulfhydryls in α_2 and β_2 subforms were obtained by a procedure previously described (D'Alessio et al., 1975). Selectively deamidated β subunit and fully reduced and carboxymethylated α_2 isoenzyme were prepared as already reported (Di Donato et al., 1986). Fully reduced and denatured α_2-BS-RNase was obtained as in Parente and D'Alessio (1985).

Separation of Peptides. Peptides were separated by RP-HPLC, on Ultrasphere ODS columns (Beckman), by using the elution conditions described in Di Donato et al. (1986).

Enzymatic Methyl Esterification Assay. Enzymatic methyl esterification was performed essentially as reported by Di Donato et al. (1986) with minor modifications. Briefly, 60 μL of the incubation mixture contained 8 μL of methylation buffer [citrate/phosphate/EDTA buffer, pH 6.2, prepared according to Kim and Paik (1970)], 16 μg of PCMT from bovine brain (sp act. 1700 units/mg), 5 nmol of [*methyl*-^{14}C]AdoMet (sp act. 100–150 cpm/pmol, Amersham International, Bueks, U.K.), and 0.6 nmol of peptide substrate, unless otherwise specified. The incubation, carried out at 37 °C, was stopped by addition of 60 μL of 0.5 M borate buffer, pH 10. The [^{14}C]methyl incorporation into substrates was evaluated by extracting with isoamyl alcohol the methanol released after alkali treatment.

Preparation of Cyclic Imide (IM) and Methyl Ester (ME) Derivatives of Peptide α16. The cyclic imide derivative of peptide α16 was prepared by incubating peptide α16 (10 μM) in methylation buffer in the presence of AdoMet (87.6 μM) and PCMT (749 units/mL), at 37 °C. After 60 min, the reaction was stopped by the addition of an inhibitory concentration of AdoHcy (1.1 mM) and left at 37 °C for 30 min. The sample was then applied on a RP-HPLC column, and the fractions containing the cyclic imide were collected (see Figure 2) and lyophilized.

The methyl ester derivative of peptide α16 was prepared with the same procedure, except that the incubation buffer was 50 mM pyridinium acetate, pH 6.2, and radioactive [*methyl*-^{14}C]AdoMet (sp act. 100 cpm/pmol) was used. After the addition of AdoHcy the reaction mixture was immediately lyophilized.

Kinetics of Demethylation of the Methyl Ester Derivative of Peptide α16. The [^{14}C]methyl ester derivative of peptide α16 was dissolved in the appropriate buffer system and incubated at 37 °C. The extent of demethylation was evaluated from the remaining methyl ester by determining the [^{14}C]-methanol extracted by isoamyl alcohol after alkali treatment. Nonspecific radioactivity extracted with isoamyl alcohol was evaluated by running a parallel experiment in which the sample was the reaction mixture used for the preparation of the methyl ester derivative of peptide α16, with the addition of an inhibitory concentration of AdoHcy in order to prevent methyl ester formation.

Treatment of Kinetic Data. The kinetic constants were derived from the corresponding half-life values. The value of k_{hydr}, i.e., the rate constant for the hydrolysis of the cyclic imide derivative of peptide α16, was used to calculate the values of k_3 and k_4 (see Figure 1 and Table II) with the following equations: $k_3 + k_4 = k_{hydr}$ and $k_3 = {}^7/_3 k_4$ (Aswad, 1985).

Other Methods. Hydroxylamine cleavage, trypsin hydrolysis, and amino acid analyses were as already described (Di Donato et al., 1986). Renaturation experiments were carried out as described by Parente and D'Alessio (1985). Ribonuclease activity was assayed with the method of Kunitz (1946) in the same buffer of the renaturation experiment. Purification of AdoMet was as previously described (Zappia et al., 1969).

Results

Methyl Esterification of Seminal RNase Peptides. We have previously shown that peptide α14, corresponding to the sequence 63–76 of BS-RNase, and containing an iso-Asp residue at position 67, acts as a methyl acceptor in the reaction

Table I: Enzymatic Methyl Esterification of BS-RNase Peptides[a]

peptide	sequence	methyl group incorporation (mol/mol)
	67 76	
β11	N-G-Q-T-N-C-Y-Q-S-K	0.01
	67 76	
α11	D-G-Q-T-N-C-Y-Q-S-K	0.09
	63 76	
α14	V-T-C-K-D^{-}G-Q-T-N-C-Y-Q-S-K	0.74
	63 76	
α15	V-T-C-K-D-G-Q-T-N-C-Y-Q-S-K	0.11
	62 76	
α16	K-V-T-C-K-D^{-}G-Q-T-N-C-Y-Q-S-K	0.75
	62 76	
α17	K-V-T-C-K-D-G-Q-T-N-C-Y-Q-S-K	0.12

[a] D^{-} indicates an aspartyl residue linked to the following residue by an isopeptide bond.

catalyzed by PCMT, whereas peptide α15, with an identical sequence, but with a normal Asp residue at position 67 (see Table I), is not methylated by the enzyme (Di Donato et al., 1986).

We extended these observations by studying as substrates of PCMT all the peptide segments of BS-RNase including sequence position 67. Table I shows the methyl-accepting ability of these peptides: peptides α11, α15, and α17, containing a normal peptide bond between residues Asp^{67}–Gly^{68}, and peptide β11, with an Asn residue at position 67, are not substrates of the enzyme, whereas peptides α14 and α16 are methylated to a near-stoichiometric extent. The low methyl incorporation observed for peptides α15 and α17 is likely due to contamination from the corresponding iso-Asp-containing peptides α14 and α16 during the chromatographic separation by RP-HPLC (Di Donato et al., 1986).

Methyl esterification of peptide α16 was studied under initial velocity conditions with a peptide concentration ranging from 0.46 to 6.6 μM. From a Lineweaver–Burk plot ($R > 0.999$) of the kinetic data an apparent K_m of 6.17 μM and a V_{max} of 19.56 nmol min^{-1} mg^{-1} were determined. These values are of the same order of magnitude of those reported for other iso-aspartyl-containing substrates of PCMT (Murray & Clarke, 1984; Johnson & Aswad, 1985; Aswad et al., 1987).

Conversion of the Isopeptide Bond of Peptide α16 into a Normal Peptide Bond. The following experiments were designed in order to investigate the possibility that enzymatic methyl esterification of peptide α16, which contains an isopeptide linkage, can induce the regeneration of the normal peptide bond, through the sequence of steps shown in Figure 1. This would in fact lead to the conversion of peptide α16 into peptide α17 (see Table I).

In the first experiment 15 nmol of peptide α16 was incubated at 37 °C with PCMT (900 units) in the presence of *S*-adenosyl-L-[*methyl*-^{14}C]methionine (87.6 μM, sp act. 26 cpm/pmol) in a final volume of 1.25 mL of methylation buffer. The formation of the reaction products at different time intervals was analyzed by RP-HPLC. In the samples not immediately subjected to chromatography the reaction was stopped by addition of an equal volume of chromatographic starting eluent, followed by freezing at −20 °C. Figure 2 shows the elution profiles of the samples at the indicated incubation times. Enzymatic methylation produced the disappearance of peptide α16 and the appearance of two new peaks marked IM and ME (Figure 2A). The material collected at each time interval as peptide ME, eluting at 48 min, was divided into

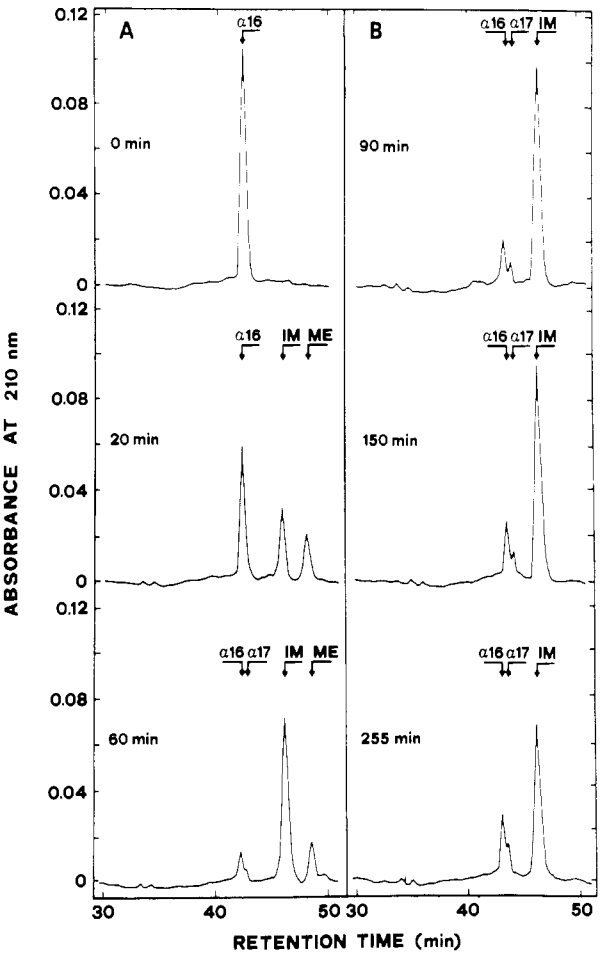

FIGURE 2: RP-HPLC profiles of the reaction products of the enzymatic methylation of peptide α16 in the absence (A) and in the presence (B) of an inhibitory concentration (1.1 mM) of AdoHcy added after 60 min of incubation. ME and IM are the methyl ester derivative and the succinimidyl derivative, respectively, of peptide α16.

two aliquots. One was subjected to radioactivity measurement while the other was alkali treated under conditions for selective hydrolysis of methyl esters, in order to measure the extent of methyl esterification. The results indicated that all the radioactivity associated with peak ME was base-labile and was entirely recovered as [^{14}C]methanol. Moreover, the incorporation of [^{14}C]methyl groups was found to be stoichiometric with the peptide. Thus peak ME could be identified as the primary product of the methylation reaction, i.e., the [^{14}C]-methyl ester derivative of peptide α16.

Peptide IM, which represented the major molecular species after 60 min, contained no radioactivity. It was identified as the cyclic imide derivative of peptide α16 after the following experiments. An aliquot of the eluted material was subjected to alkaline hydrolysis in 0.5 M borate buffer, pH 10, for 180 min at 37 °C. This treatment would open a cyclic succinimide, producing the two isomeric peptides containing an aspartyl and an isoaspartyl residue, respectively. Figure 3B shows the chromatographic pattern of the products of alkaline hydrolysis of peptide IM separated by RP-HPLC. Their elution times coincide with those of peptides α16 (with iso Asp^{67}) and α17 (with Asp^{67}), respectively.

Peptide IM was also reacted with hydroxylamine (Bornstein & Balian, 1977), and the reaction mixture after 120 min of incubation was fractionated by RP-HPLC as is shown in Figure 3C. The separated peptides were identified by their amino acid compositions determined after acid hydrolysis. They were found to correspond to the sequence regions 62–67

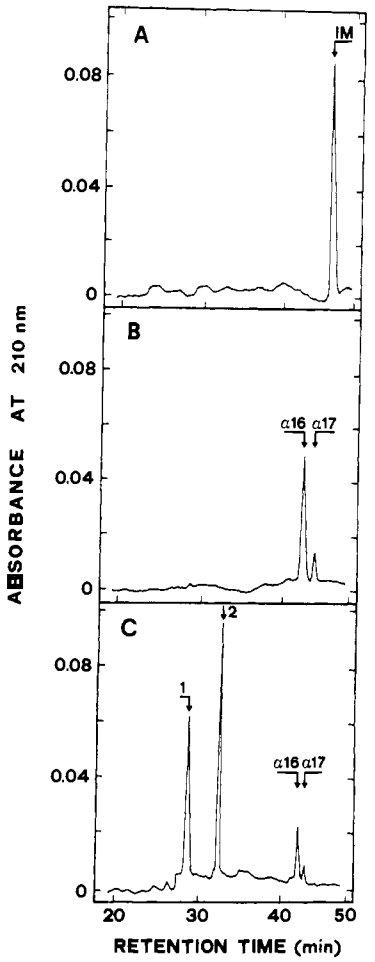

FIGURE 3: RP-HPLC profiles of the products of incubation of peptide IM before (A) and after the addition of borate buffer (B) and hydroxylamine (C).

(peptide 1) and 68–76 (peptide 2) of peptide α16. These products were those expected for the cleavage of a succinimide ring linking the residues at positions 67 and 68 of peptide α16 (see Table I and Figure 1). It should be noted that peptides α16 and α17 (the products of hydrolysis of the imide-containing peptide, see Figure 3B) are also present as side products of the reaction in the HPLC pattern illustrated in Figure 3C. This is not surprising, given the alkaline pH of the hydroxylamine reaction.

The identification of peptides IM and ME as the imide-containing and the methyl ester derivatives of the original isopeptide α16, respectively, allowed a clear interpretation of the experiment illustrated in Figure 2A: (1) peptide α16 is enzymically methylated at the α-COOH of isoaspartyl-67; (2) the newly produced methyl ester spontaneously demethylates, generating a cyclic succinimide derivative.

These results could suggest that the succinimidyl peptide is the final product of the process, this excluding the possibility of spontaneous hydrolysis of the imide with the regeneration of the original peptide α16 and the production of peptide α17. In order to investigate the fate of the succinimidyl derivative of peptide α16, the experiment illustrated in Figure 2A was repeated, adding after 60 min of incubation AdoHcy (1.1 mM) to the reaction mixture. This addition would inhibit any further methyl esterification of the α-carboxyls of iso-Asp[67], thus revealing whether hydrolysis of the imide actually occurred. The results of the experiment illustrated in Figure 2B show that inhibition of PCMT produced the disappearance of newly formed peptide IM, which converted into two peptides, identified by their retention times as peptides α16 and

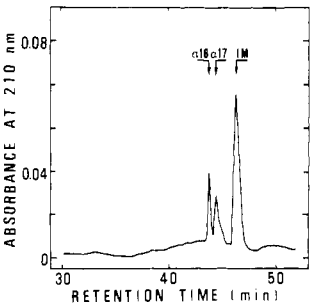

FIGURE 4: RP-HPLC profile of the reaction products of peptide α16, PCMT, and AdoMet, after a prolonged incubation (12 h).

α17, respectively, in a ratio of 7:3.

These results are in line with the results of McFadden and Clarke (1987), thus supporting the proposal that isoaspartyl peptides can be repaired through a series of reactions in which the reaction catalyzed by PCMT is the only enzymatic step.

In order to further test this hypothesis, we examined the effect of a prolonged incubation of peptide α16 in the presence of the methylating system. Peptide α16 (3.7 nmol) was incubated in methylation buffer for 12 h at 37 °C in the presence of *S*-adenosyl-L-[-*methyl*-^{14}C]methionine (87.7 μM, sp act. 26 cpm/pmol) and PCMT (280 units) in a final volume of 360 μL. In order to ensure an effective rate of methylation even after an extended incubation, adenosine deaminase from *A. oryzae* (37 units) was added to the reaction mixture. This would remove any inhibitory AdoHcy formed during the incubation, by converting it into *S*-inosylhomocysteine, which lacks any inhibitory effect (Oliva et al., 1980a,b). Figure 4 shows the chromatographic separation of the reaction products after 12 h of incubation. Peptides IM, α16, and α17 were identified by their retention times. Peptides α16 and α17 were found to be in a ratio 4.5:3. Thus, after prolonged incubation with the methylating system, the ratio between peptides α16 and α17 was significantly lower than that observed at shorter incubation times (7:3; see Figure 2B). This could only be explained if an effective repair of isoaspartyl-containing peptide α16 had occurred, through consecutive cycles of methylation–demethylation, producing increasing amounts of peptide α17. This conclusion was confirmed by the finding that 8.98 mol of [^{14}C]methanol was produced during the 12 h of incubation, i.e., more than twice the amount of the methylatable peptide α16.

Stability of the Cyclic Imide and of the Methyl Ester Derivatives of Peptide α16. The stability of the cyclic imide and of the methyl ester derivatives of peptide α16 was investigated by incubating the peptide derivatives in citrate/phosphate/EDTA buffer, pH 6.2, or in 0.25 M sodium phosphate buffer, pH 7.4, at 37 °C.

The kinetics of ring opening of the succinimidyl derivative of peptide α16 (Figure 5, lower panel) were followed by withdrawing aliquots at increasing time intervals and evaluating the residual amount of cyclic imide by integration of the peak obtained after chromatographic separation by RP-HPLC.

The kinetics of demethylation of the methyl ester derivative (see Figure 5, upper panel) were followed by measuring the residual content of base-labile methyl groups as described under Experimental Procedures. The kinetic constants of the reaction steps as outlined in Figure 1 are listed in Table II.

Methylation Esterification of Native, Monomeric, and Denatured BS-RNase. On the basis of the results obtained with the peptides containing sequence position 67 of BS-RNase α-type subunit, we tested the methyl-accepting capability of the same sequence when it is part of the whole protein structure. Therefore, the various molecular forms of BS-

Table II: Kinetic Constants of Methylation and Demethylation of Peptide α16 from α Subunit of BS-RNase[a]

reaction step		pH 6.2	pH 7.4
1	V_{max}	19.6 nmol min^{-1} mg^{-1}	
	K_m	6.17 μM	
2	k_2	12.61 × 10^{-3} min^{-1}	240 × 10^{-3} min^{-1}
3	k_3	0.65 × 10^{-3} min^{-1}	7.5 × 10^{-3} min^{-1}
4	k_4	0.28 × 10^{-3} min^{-1}	3.2 × 10^{-3} min^{-1}

[a] Kinetic constants refer to the reaction scheme of Figure 1.

Table III: Enzymatic Methyl Esterification of Isoenzymes of BS-RNase and Their Monomeric Derivatives

protein	concentration (μM)	methyl group incorporation (mol/mol)
α_2-BS-RNase	166	0.01
αβ-BS-RNase	166	0.01
β_2-BS-RNase	166	0.02
α-M$(CM)_2$-BS-RNase	166	0.03
β-M$(CM)_2$-BS-RNase	166	0.01
α-M$(CM)_{10}$-BS-RNase	28.8	0.66[a]
α-M$(SH)_{10}$-BS-RNase	28.0	0.78[a]

[a] Calculated by assuming a ratio 7:3 for isoaspartyl:aspartyl bonds.

RNase were assayed as substrates of PCMT. Table III shows the results of the experiments of incorporation of methyl groups by the isolated isoenzymatic forms of native BS-RNase. As none of the dimeric forms of the enzymes were substrates for PCMT (see Table III), we tested also the methyl-accepting capacity of the catalytically active monomeric derivatives of the enzyme, prepared by selective reduction and carboxymethylation at half-cystine residues 31 and 32 (D'Alessio et al., 1975). The two monomeric derivative forms of BS-RNase, i.e., α-M$(CM)_2$-BS-RNase and β-M$(CM)_2$-BS-RNase, were not substrates for the carboxylmethylation reaction (see Table III).

We found that BS-RNase became a stoichiometric substrate for carboxyl methyl esterification (see Table III) only after the unfolding of its tertiary structure. This was obtained by reducing both the inter- and intrasubunit protein disulfides, under denaturing conditions. The K_m and V_{max} values of the methylation reaction were found to be 13.1 μM and 1.83 nmol min^{-1} mg^{-1}, respectively. The fully reduced and denatured protein with the sulfhydryls blocked by alkylation with iodoacetate was also a good substrate (see Table III).

These data lead to the conclusion that the tertiary structure of BS-RNase hinders the methyl esterification of the isoaspartyl residues present in α subunit.

To further confirm these results, native, catalytically active α-M$(CM)_2$-BS-RNase was unfolded by completely reducing its intrachain disulfide bridges under denaturing conditions. The unfolding of the protein three-dimensional structure, monitored by the loss of ribonuclease activity (from 595 to 0 units/mg), was paralleled by the appearance of methyl-accepting capability (from 0.015 to 0.57 mol/mol). The unfolded protein, freed of the denaturing and reducing reagents, was refolded by air reoxidation of the reduced disulfides, at pH 8.0, 25 °C, for 24 h. The refolding of the protein structure restored all the ribonuclease activity (604 units/mg), while the methylation site appeared no longer susceptible to PCMT action, with a methyl-accepting capacity of 0.03 mol/mol.

Discussion

For several years methylation of proteins and peptides by eucaryotic protein methyltransferases has not been fully understood in its physiological significance, as it appeared to be largely substoichiometric and nonselective with regard to the protein substrate. The observation of Aswad (1984) and

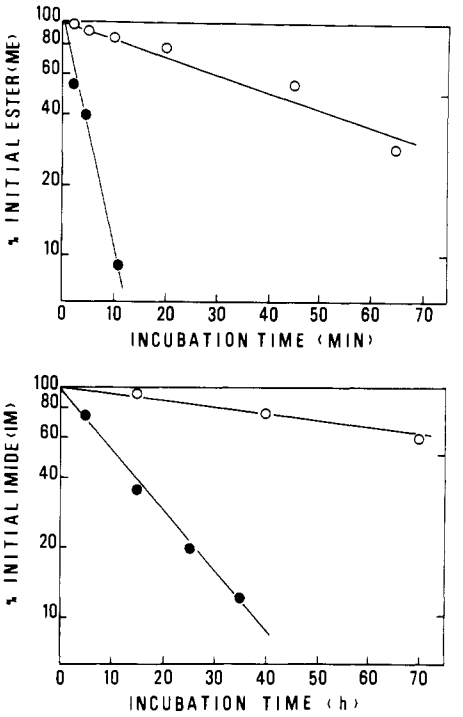

FIGURE 5: Kinetics of demethylation of the methyl ester derivative of peptide α16 (ME) (upper panel) and of the ring opening of the succinimidyl derivative of peptide α16 (IM) (lower panel) at pH 6.2 (○) and 7.4 (●).

Murray and Clarke (1984) showed that substoichiometric methylation and lack of selectivity were only apparent. In fact, the reaction catalyzed by eucaryotic protein carboxyl methylases occurs selectively at the free α-carboxyl functions of isoaspartyls, i.e., aspartic acid residues linked with their β-COOH, through an unusual isopeptide bond, to the adjacent residue, which is generally a glycine (Aswad, 1984; Murray & Clarke, 1984; Di Donato et al., 1986).

The chemical mechanism for the generation of isopeptide bonds in proteins and peptides has also been elucidated. Isoaspartyl residues are produced through deamidation of asparaginyl residues in Asn-Gly sequences (Bornstein & Balian, 1974). This occurs via a cyclic succinimide intermediate which upon spontaneous hydrolysis opens on either side of the imide nitrogen, generating a mixture of aspartyl and isoaspartyl residues (Aswad, 1984; Murray & Clarke, 1984; Di Donato et al., 1986).

So far, the knowledge of the chemical events, i.e., the selective deamidation of proteins and the enzyme-catalyzed methylation of the deamidated residues, has not produced an immediate understanding of the physiological relevance of these processes. Selective deamidation has been previously related to the aging of proteins (Robinson & Rudd, 1974). Moreover, an increase in the methyl-accepting capacity of proteins in aged erythrocytes has suggested a correlation between deamidation and methylation in aged proteins (Galletti et al., 1983). Recently, isopeptide bonds have been proposed to originate as errors in protein synthesis (Clarke, 1985).

A more direct hypothesis has been advanced, suggesting that methylation at the α-carboxyl functions of isoaspartyl residues constitutes a system for the "repair" of altered aged proteins (Murray & Clarke, 1984). The results of McFaden and Clarke (1987) are in line with this hypothesis, as they demonstrate the conversion of chemically generated isotetragastrin into the usual tetragastrin.

The data presented in this paper, obtained with a peptide segment derived from the naturally deamidated α-type subunit

of BS-RNase, confirm that the proposed repair mechanism is operative but may be restricted by the three-dimensional structure of the protein substrate.

Peptide α16, from α subunit of BS-RNase, is recognized and near stoichiometrically methylated by PCMT with a K_m in the range of values previously determined for other isoaspartyl-containing peptides (McFadden & Clarke, 1986, 1987). The product of the enzymatic reaction, i.e., the methyl ester derivative of peptide α16, rapidly demethylates, yielding the cyclic imide derivative of peptide α16, with a rate constant of 240×10^{-3} min^{-1} at pH 7.4 (see Table II), which is very similar to that reported for the demethylation of methyliso-ACTH (Johnson & Aswad, 1985). It is noteworthy that the rate constant reported for the same demethylation process of methylated isotetragastrin is an order of magnitude higher (McFadden & Clarke, 1987). This would suggest that this reaction is influenced by the primary structure of the peptide.

The cyclic imide derivative of peptide α16 is transiently stable. The kinetic constants of ring opening k_3 and k_4 are much lower than k_2 (see Figure 1 and Table II), which indicates that ring opening is the rate-limiting step in the repair process. It is surprising that, in contrast with the preceding step, hydrolysis of the cyclic imide is not influenced by the surrounding amino acid residues, as comparable rate constants have been determined for peptide α16, ACTH (Johnson & Aswad, 1985), and tetragastrin (McFadden & Clarke, 1987).

The amount of "repaired" peptide, in which the isopeptide bond has been eliminated, generated by the opening of the imide ring can be as much as 42% of the original peptide containing the unusual isopeptide bond, after 12 h at pH 7.4 and 37 °C. This is due to the lack of reactivity of the Asp-containing peptide, while the residual isopeptide bond can undergo cycles of methylation–demethylation, with the possibility of an eventual total "repair" of the altered isopeptide.

However, the chemical feasibility of the "repair" hypothesis gives no clue to a general physiological significance of the repair process, as several proteins may not be substrates of PCMT, even though they contain isoaspartyl residues. This conclusion can be drawn from the observation that subforms α_2, $\alpha\beta$, β_2, and the isolated α subunit of BS-RNase, a rare case of naturally deamidated protein, are not methylated by PCMT and thus cannot be repaired by the mechanism described above. In fact, α subunit becomes stoichiometrically methylatable by PCMT only after it is completely unfolded. It should be considered that our experimental conditions, which are optimal for protein methyl esterification, may not mimic the in vivo conditions. However, this result strongly suggests that the tertiary structure of an isoaspartyl-containing protein is a major determinant for a productive enzyme–substrate interaction. This conclusion may be of a general value, as also in other proteins, such as RNase A (Ciardiello et al., unpublished results) and EGF (Galletti et al., 1987), isoaspartyl residues are not methylated unless the protein is fully unfolded. It should be noted that in all these proteins intrachain disulfides are present. Interestingly, other isoaspartyl-containing proteins with no disulfide bridges, i.e., ACTH (Aswad, 1984) and calmodulin (Johnson et al., 1987), are substrates for PCMT. This may be interpreted in terms of an additional stability, conferred to the protein structure, which hinders the interaction of the methylatable site with the enzyme.

Registry No. RNase, 9001-99-4; Asp, 56-84-8; protein carboxyl *O*-methyltransferase, 9055-09-8.

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