Effect of Methylation on Susceptibility of Protein to Proteolytic Enzymes†

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ABSTRACT: Pancreatic ribonuclease was reductively methylated with formaldehyde and sodium borohydride. The reaction was highly specific for the ϵ-NH_2 group of lysine residues, and the products were identified as monomethyl- and dimethyl-substituted lysine derivatives. The composition of these methylated lysine derivatives in ribonuclease was dependent on the amount of formaldehyde used. At the highest concentration of formaldehyde, approximately 80% of the ϵ-NH_2 groups in the protein was substituted with methyl groups. Contrary to the commonly held notion, trypsin and α-chymotrypsin hydrolyzed both the methylated and the native ribonuclease at equal rates. Identical results were also obtained with polylysine and calf thymus arginine-rich histone which were reductively methylated.

Methyl-substituted amino acids have been found in proteins as well as in the free state, and a few *S*-adenosylmethionine–protein methyltransferases have been identified (Paik and Kim, 1971). However, the biological significance of protein methylation remains obscure. Since methyl substitution changes the charge of the side chain, it has been suggested that methyl substitution of the ϵ-NH_2 group of lysine residues in a protein molecule protects the protein from proteolytic attack by intracellular enzymes. Consistent with this hypothesis the esterase activity of pancreatic trypsin on L-lysine methyl ester was greatly depressed when the hydrogen of the ϵ-NH_2 group of L-lysine was replaced with methyl group (Gorecki and Shalitin, 1967; Benoiton and Deneault, 1966). In order to test this hypothesis of methylated lysine residues at the level of protein molecules, we have chosen pancreatic ribonuclease as model compound. The ribonuclease was methylated reductively with formaldehyde and sodium borohydride. Even though over 80% of the ϵ-NH_2 groups in ribonuclease molecule were methylated, as judged by the decrease in ninhydrin or trinitrobenzenesulfonic acid reaction, the susceptibility of the methylated ribonuclease to the action of trypsin or α-chymotrypsin was identical with that of the native protein. The same results were obtained with reductively methylated polylysine and histone.

Materials and Methods

Materials. Five-times-crystallized pancreatic bovine ribonuclease, pancreatic trypsin, and α-chymotrypsin, polylysine (approximate molecular weight 80,000), and calf thymus arginine-rich histone have been purchased from Sigma Chemical Co. Formaldehyde, sodium borohydride, ninhydrin, and 2,4,6-trinitrobenzenesulfonic acid were obtained from Fisher Scientific Co. These chemicals were used without further purification. The remainder was obtained from various local sources.

Reductive Methylation of Protein. Reductive methylation of protein was carried out by a slight modification of the method published previously (Means and Feeney, 1968); 9 ml of protein suspension containing 30 mg of protein in 0.07 M borate buffer (pH 9.0) and 3 mg of sodium borohydride were introduced into a test tube (1.6 × 15 cm) in ice. Ten microliters of 5 M formaldehyde (18.5%) diluted in the above buffer was added to the tube and mixed. Ten microliters of the formaldehyde solution was then added at 5-min intervals. After completion of the reaction, the mixture was left for an additional 5 min in ice, and the contents were dialyzed against 4000 ml of water at 3° for 5 hr, and for another 12 hr after changing the water.

Ninhydrin Reaction. The ninhydrin reaction was carried out by the method of Moore and Stein (1948); Coleman colorimetric tubes (1.8 × 10.2 cm) are charged with 1.0 ml of an aqueous solution to be tested and 1.0 ml of ninhydrin solution (mix 30 ml of 0.2 M citrate buffer (pH 5.0), 30 ml of 4% ninhydrin in ethyl Cellosolve, and 1 ml of $SnCl_2$ suspension containing 50 mg). The tubes were heated in a boiling water for 5 min. After cooling the tubes in cold water for a few minutes, 5 ml of water was added and absorbancy at 580 mμ was read.

2,4,6-Trinitrobenzenesulfonic Acid Reaction. In addition to ninhydrin reaction, the amounts of amino groups were also determined by using 2,4,6-trinitrobenzenesulfonic acid according to the procedure of Habeeb (1966); 1 ml of 4% sodium bicarbonate buffer (pH 8.5) was added to 1 ml of protein solution. After adding 1 ml of 0.1% of trinitrobenzenesulfonic acid the solution was allowed to react at 37° for 2 hr. One milliliter of sodium dodecyl sulfate was then added to solubilize the protein and prevent its precipitation on addition of 0.5 ml of 1 N HCl. The absorbancy at 335 mμ was determined against a blank treated as above but with 1 ml of water instead of the protein suspension.

Biuret Reaction. In order to determine the amount of polylysine (the determination of protein concentration of polylysine with the method of Lowry *et al.* (1951) is not applicable because of lack of tyrosine), the biuret reaction was employed; 3 ml of protein suspension was reacted with an equal volume of biuret reagent. The biuret reagent was prepared as follows. $CuSO_4$ (1.5 g) and sodium potassium tartarate (6.0 g) were dissolved in 500 ml of water. Three-hundred milliliters of 10% NaOH was added into the above solution while constantly stirring, and the final volume was adjusted to 1000 ml

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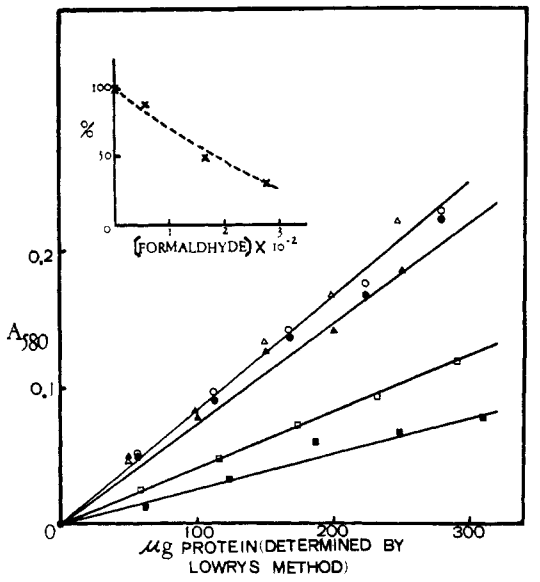

FIGURE 1: Determination of ninhydrin color of ribonuclease reductively methylated with varying amounts of formaldehyde. Since reductively methylated ribonuclease has to be dialyzed before use and dialysis changes the volume, the concentration of protein was determined after dialysis by the method of Lowry *et al.* (1951). Detailed procedure for reductive methylation is described under Methods. (○) indicates control ribonuclease which has been dialyzed simultaneously with the assay; (●) for the sample treated with only 2.75×10^{-2} M of HCHO; (△) for sample treated with 0.88×10^{-2} M of $NaBH_4$ alone; the remaining samples were treated with constant amount of $NaBH_4$ (0.88×10^{-2} M) with varying amounts of HCHO, (▲) with 0.55×10^{-2} M, (□) with 1.65×10^{-2} M, and (■) with 2.75×10^{-2} M.

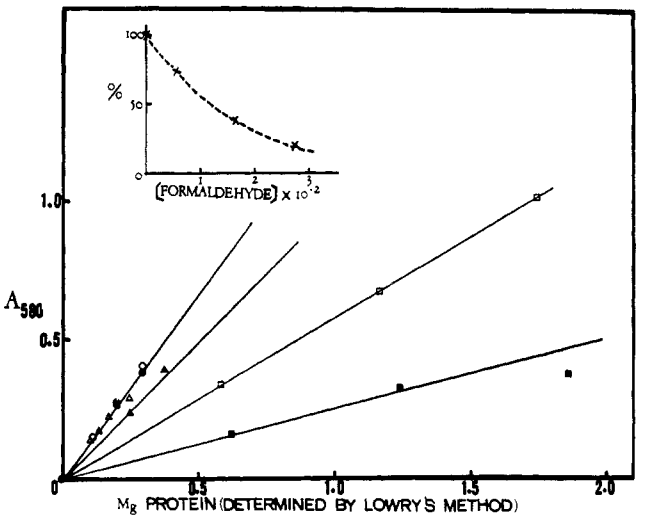

FIGURE 2: Determination of 2,4,6-trinitrobenzenesulfonic acid reaction of ribonuclease reductively methylated with varying amounts of formaldehyde. The conditions are identical with those in Figure 1.

with water. The reagent-treated protein suspension was left for 30 min at room temperature and the absorbancy at 540 mμ was determined against a blank treated as above with 3 ml of water.

Oxidation of Ribonuclease. Oxidation of ribonuclease with performic acid was carried out essentially as described by Hirs (1956). Preformic acid was prepared by mixing 9.6 ml of formic acid and 0.5 ml of H_2O_2, and was allowed to stand at room temperature for 2 hr in a stoppered flask. The oxidation was carried out in a flask with a side arm. In the main compartment of the flask 400 mg of ribonuclease in 10 ml of formic acid was added; and in the side arm 5.0 ml of freshly prepared performic acid. The flask was cooled for 30 min in ice and the contents of the side arm tipped into the main compartment. After 2.5 hr in ice, the contents were transferred quantitatively into 800 ml of precooled distilled water, and the diluted solution was immediately lyophilized. The lyophilized sample was dissolved in 160 ml of water and was again lyophilized.

Tryptic Hydrolysis of Protein. Three-tenth milliliter of protein suspension (200 μg of methylated or native) and one-tenth milliliter of 0.5 M phosphate buffer (pH 7.2) were preincubated in Coleman colorimetric cuvet (1.8 × 10.2 cm) for 3 min at 37°. The reaction was initiated with the addition of 0.1 ml of trypsin (200 μg for nonoxidized ribonuclease and polylysine, 10 μg for oxidized ribonuclease, and 0.5 μg for histone). After the indicated period of time, 0.5 ml of 0.2 M cold citrate buffer (pH 5.0) was added and the tube was immediately placed in ice to stop the reaction. One milliliter of ninhydrin solution was then added to the tube and the color reaction was carried out according to the procedure described above. The values in the following experiments were corrected for blank value. The blank was prepared by incubating the mixture as the assay in the absence of trypsin for the indicated period of time. Citrate buffer and ninhydrin solution were added, followed by the addition of trypsin. The remaining procedure was the same as the assay. The tube in which trypsin alone was incubated in the phosphate buffer for 3 hr did not change the ninhydrin color from the fresh trypsin value.

Assay of Ribonuclease Activity. Assay for pancreatic ribonuclease was essentially as described by Kalnitsky *et al.* (1959); 0.1 ml of ribonuclease solution was incubated with 1.9 ml of 0.1 M sodium phosphate buffer (pH 7.3) and 1.0 ml of 2% (w/v) RNA for 4 min at 30°. The reaction was terminated by adding 1.0 ml of 0.75% uranyl acetate in 25% (w/v) $HClO_4$. After complete mixing, the mixture was centrifuged, and 0.1 ml of the supernatant was diluted with 3 ml of water and absorbancy of the acid-soluble oligonucleotides was determined at 260 mμ.

Amino Acid Analysis. The extent of methylation of the ε-NH_2 group of lysine residues in the protein by reductive methylation was followed by analyzing the HCl hydrolysate of the protein. A portion of the protein was hydrolyzed in 6 N HCl at 110° for 48 hr in a vacuumed tube, the hydrolysate was concentrated under reduced pressure, and HCl was removed by repeated washing with water. The residue was dissolved in appropriate amount of water, and a portion was analyzed by the Beckman automatic amino acid analyzer by the elution system described previously (Paik and Kim, 1970a,b). Relative molar ninhydrin color values of 1.00, 0.881, and 0.822 for lysine, ε-*N*-monomethyllysine and ε-*N*,ε-*N*-dimethyllysine, respectively, were taken from published data (Means and Feeney, 1968). Finally, the concentration of protein (except polylysine) was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Results

Extent of Reductive Methylation of Ribonuclease. Figures 1 and 2 illustrate the extent of modification of the free amino groups in the ribonuclease molecule by reductive methylation.

TABLE I: Extent of Methylation of Ribonuclease, as Judged by Amino Acid Analysis and Enzyme Activity.

Ribonuclease Treated with ($\times 10^{-2}$ M)		RNase Act.	% in		
HCHO	$NaBH_4$	(%)	L[a]	MML	DML
0	0	100.0	100.0	0	0
2.75	0	75.7	100.0	0	0
0	0.88	96.8	100.0	0	0
0.55	0.88	67.8	84.6	13.1	2.2
1.65	0.88	5.6	53.3	17.5	29.2
2.75	0.88	1.0	22.1	11.6	66.3

[a] L is used for lysine, and MML and DML for ϵ-*N*-monomethyllysine and ϵ-*N*,ϵ-*N*-dimethyllysine, respectively.

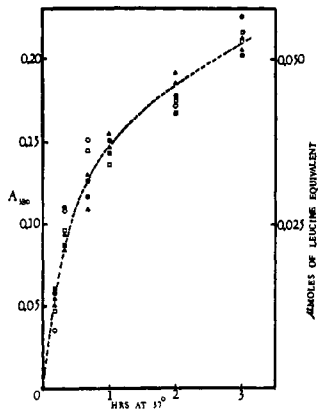

FIGURE 3: Rate of tryptic hydrolysis of methylated ribonuclease. All the symbols represent ribonuclease treated in identical way as in Figure 1. Trypsin (200 μg) and ribonuclease (200 μg) preparations were used. Detailed procedure for incubation and other assay conditions are described under Methods. The blank value of the control was 0.510 and the highly methylated 0.354 at 580 mμ in Coleman spectrophotometer. Composition of various lysine derivatives in preparations are listed in Table I.

Both the ninhydrin and the 2,4,6-trinitrobenzenesulfonic acid reaction indicate that approximately 80% of the free amino groups were modified. Furthermore, the figures show that the extent of modification is formaldehyde dependent. Concomitant with the modification of the molecule, ribonuclease activity decreased greatly, the latter exceeding the former (Table I). The fact that the rate of enzymatic inactivation exceeds the rate of modification was also noted by Means and Feeney (1968). When these modified proteins were hydrolyzed in HCl and were analyzed on the amino acid analyzer, lysine was the only amino acid visibly modified. Although not shown, these modified lysine derivatives were identified as ϵ-*N*-monomethyllysine and ϵ-*N*,ϵ-*N*-dimethyllysine. They were eluted simultaneously on the amino acid analyzer column with authentic ϵ-*N*-monomethyllysine and ϵ-*N*,ϵ-*N*-dimethyllysine (Paik and Kim, 1970a,b). Table I lists the relative amounts of lysine, ϵ-*N*-monomethyllysine, and ϵ-*N*,ϵ-*N*-dimethyllysine in ribonuclease molecule which were treated with varying amounts of formaldehyde. With low concentration of formaldehyde, ϵ-*N*-monomethyllysine is formed in relatively high amount. However, increasing the concentration of formaldehyde increases the relative amount of ϵ-*N*,ϵ-*N*-dimethyllysine. At the highest concentration of formaldehyde (2.75×10^{-2} M), approximately 80% of the lysine residues are methylated, confirming the results obtained with the ninhydrin and the 2,4,6-trinitrobenzenesulfonic acid reactions (Figures 1 and 2).

Susceptibility of Methylated Ribonuclease to Trypsin. The ribonuclease preparations methylated in the preceeding experiments were subjected to the action of pancreatic trypsin. As seen in Figure 3, although there is some scattering of the points, the overall rate of tryptic digestion of ribonucleases methylated to various extents is about the same. Extending the incubation period up to 6 hr did not increase the ninhydrin color value (A_{580}), reaching the maximum at approximately 0.21. Since 200 μg of nontreated ribonuclease alone gives an A_{580} of 0.12 and trypsin alone in the incubation mixture did not change its A_{580} value, this indicates that tryptic hydrolysis of ribonuclease increased the ninhydrin color about 80% over the original value. This increase in ninhydrin color indicates almost complete hydrolysis of susceptible bonds in ribonuclease, since 13 peptide fragments were obtained from the tryptic hydrolysis of oxidized ribonuclease and the ninhydrin color increased only about 90% (Hirs *et al.*, 1956). Since pancreatic ribonuclease contains 1 N-terminal and 8 internal lysine residues and 4 arginine residues, tryptic digestion might be expected to yield 14 or 15 peptide fragments. Therefore, the result in Figure 3 indicates that the initial rate of hydrolysis as well as the number of peptide fragments from the tryptic digestion of ribonuclease molecule is not affected by the reductive methylation of ribonuclease even though over 80% of the amino groups were modified. It should be noted that relatively large amount of trypsin was required to measure the rate of tryptic hydrolysis of ribonuclease. When oxidized ribonuclease was methylated and the protein was subjected to the action of trypsin, 10 μg of trypsin was found to bring about same rate of hydrolysis of oxidized ribonuclease as that of the nonoxidized protein (unpublished data). Under this condition, similar results were obtained for the hydrolysis of the methylated oxidized and native ribonuclease.

Susceptibility of Methylated Ribonuclease to α-Chymotrypsin. Although aromatic amino acids are not involved in

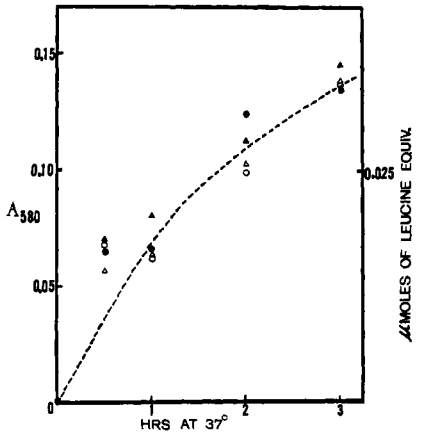

FIGURE 4: Rate of hydrolysis of methylated ribonuclease by α-chymotrypsin. (○) Represents the control, and (●), (△), and (▲) for the samples treated with 0.55×10^{-2}, 1.65×10^{-2}, and 2.75×10^{-2} M HCHO with fixed amount of $NaBH_4$ at 0.88×10^{-2} M, respectively. Ribonuclease (200 μg) and an equal amount of α-chymotrypsin were used. The blank value of the control was 0.464 and the assay 0.318 at 580 mμ. The rest of the experimental procedures are the same as in the case of trypsin.

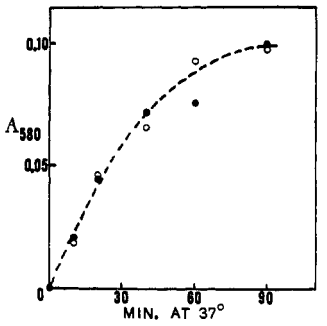

FIGURE 5: Tryptic hydrolysis of methylated polylysine. The amount of polylysine after reductive methylation and dialysis was determined by biuret reaction, since Lowry's method is not applicable. Reductive methylation of polylysine was carried out identically by the procedure for ribonuclease. Trypsin (200 μg) and polylysine (90 μg) (methylated or native) were used. Blank value of the control was 0.338 and the assay 0.324 at 580 mμ. (○) Control and (●) polylysine treated with 2.75×10^{-2} M of HCHO and 0.88×10^{-2} M of $NaBH_4$. The rest of the experimental procedures are described under Methods.

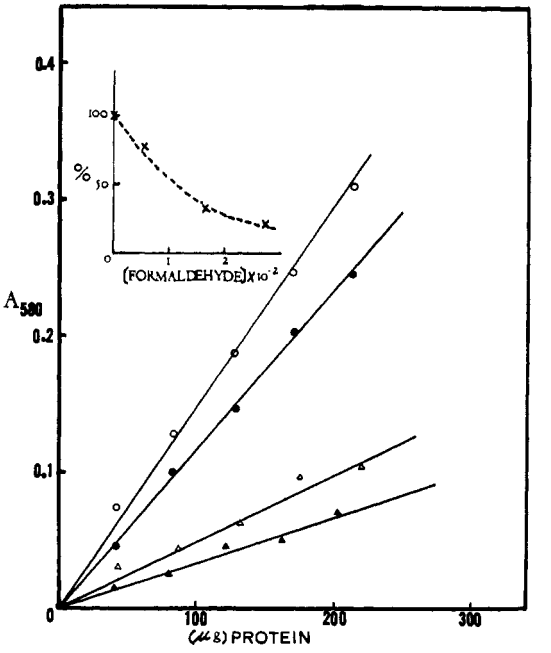

FIGURE 6: Determination of ninhydrin color of calf thymus arginine-rich histone reductively methylated with varying amounts of formaldehyde. (○) Control; (●), (△), and (▲) samples treated with 0.55×10^{-2}, 1.65×10^{-2}, and 2.75×10^{-2} M of HCHO at a fixed concentration of $NaBH_4$ (0.88×10^{-2} M), respectively. The rest of the procedures are described under Methods.

reductive methylation of ribonuclease, methyl substitution of the ϵ-NH_2 group of lysine may cause some change in conformation of the protein, thereby changing the susceptibility of ribonuclease to some other hydrolytic enzymes. Therefore, the methylated ribonuclease was subjected to the action of α-chymotrypsin. As shown in Figure 4, over 80% substitution of the ϵ-NH_2 group of lysine residues in ribonuclease molecule did not cause any change in the rate of chymotryptic digestion.

From the foregoing results, it is quite clear that methyl substitution of the ϵ-NH_2 group of lysine residues in pancreatic ribonuclease does not affect the susceptibility of the protein to the action of either trypsin or α-chymotrypsin. It was therefore considered to be worthwhile to examine the generality of this observation. Polylysine and calf thymus arginine-rich histone were chosen for this purpose.

Susceptibility of Methylated Polylysine to Trypsin. In order to examine the effect of reductive methylation of polylysine to the action of trypsin, polylysine was first reductively methylated. As shown in Table II, approximately 70% of the ϵ-NH_2 groups were modified. However, the susceptibility of this methylated polylysine to the action of trypsin was identical with that of native polypeptide (Figure 5).

Reductive Methylation of Calf Thymus Arginine-Rich Histone. Since histone contains ϵ-*N*-monomethyllysine as well as ϵ-*N*,ϵ-*N*-dimethyllysine (Murray, 1964; Paik and Kim, 1967), and an enzyme exists to methylate the ϵ-NH_2 group of lysine residues with *S*-adenosyl-L-methionine as methyl donor (Paik and Kim, 1970b), histone would be a good choice to examine it after reductive methylation in order to find some

TABLE II: Extent of Methylation of Polylysine and Arginine-Rich Histone.

Proteins	Treated with (× 10^{-2} M) HCHO	$NaBH_4$	% in L[a]	MML	DML
Polylysine	2.75	0.88	31.7	12.7	55.6
Arginine-rich histone	0	0	91.2	3.7	5.1
	0.55	0.88	89.3	6.3	4.6
	1.65	0.88	26.0	9.6	64.4
	2.75	0.88	18.3	7.1	74.6

[a] L is used for lysine, and MML and DML for ϵ-*N*-monomethyllysine and ϵ-*N*,ϵ-*N*-dimethyllysine, respectively.

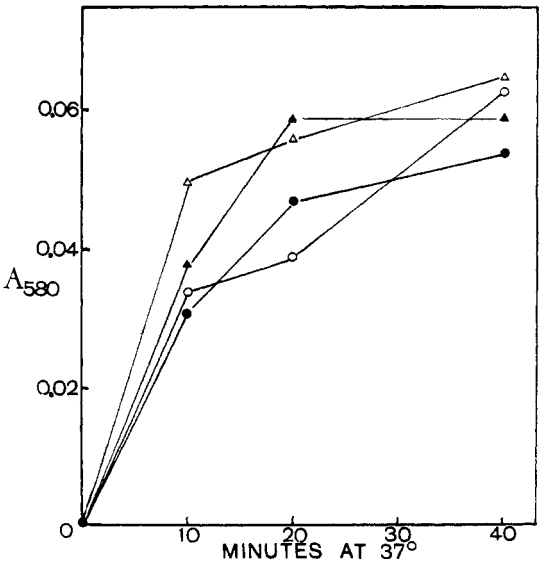

FIGURE 7: Tryptic hydrolysis of methylated arginine-rich histone. All the symbols represent the samples treated identically as in Figure 6. Trypsin (0.5 μg) and histone (100 μg) preparations were used. The blank values of the control and the sample treated with 2.75×10^{-2} M of HCHO were 0.094 and 0.078 at 580 mμ, respectively. Composition of various lysine derivatives in preparations are listed in Table II. Detailed experimental procedures are described under Methods.

physiological significance of protein methylation with respect to proteolytic action in intracellular hydrolysis. Figure 6 illustrates the extent of reductive methylation of calf thymus arginine-rich histone, determined by decrease in ninhydrin color. As in the case of ribonuclease, approximately 80% of the ϵ-NH_2 group of lysine residues were modified. This is further confirmed by amino acid analysis as shown in Table II. When the methylated histone was reacted with pancreatic trypsin (Figure 7), there was no significant difference in the rates of hydrolysis of the methylated and native histone.

Discussion

One of the main obstacles in the study of protein methylation has been the unavailability of natural substrate proteins free of methyl substitutions. Therefore, when a natural substrate protein which contained methyl groups already in the molecule was enzymatically methylated, the amount of methyl groups added by the methylase was too small to detect any effect of the methyl substitution on the protein molecule. Furthermore, enzymes which demethylate the methyl protein has not yet been found. In order to overcome this difficulty as well as to gain some guideline for the future investigation of the biological significance of protein methylation, we have used the nonenzymatic methylation of proteins because reductive methylation of protein inserts a large amount of methyl groups into the ϵ-NH_2 of lysine residues (Means and Feeney, 1968). In the present paper, a large portion of the efforts have been devoted to pancreatic ribonuclease, because of our familiarity with this enzyme (Kim and Paik, 1968, 1971) and because this enzyme has been well characterized (Sheraga and Rupley, 1962).

It has been reported by Means and Feeney (1968) that reductive methylation of approximately 80% of the ϵ-NH_2 groups in ribonuclease resulted in no significant changes in behavior during ultracentrifugal sedimentation, starch gel electrophoresis, or in the optical rotatory dispersion of the protein. Confirming the homogeneity on polyacrylamide gel electrophoresis, we have also observed no difference in p*I* values of the methylated and native ribonuclease (p*I* value was 9.10; unpublished data). Although dimethyl substitution of the ϵ-NH_2 group diminishes the basicity and monomethyl substitution increases it (Paik and Kim, 1971), the results reported above indicate that net charge of ribonuclease remains unchanged by reductive methylation. Furthermore, since the p*K* values of ϵ-NH_2, ϵ-$NH(CH_3)$, and ϵ-$N(CH_3)_2$ are in the high pH range, all these groups will be protonated at neutral pH.

Using free lysine derivatives, Gorecki and Shalitin (1967) and Benoiton and Deneault (1966) demonstrated that esterase activity of trypsin on L-lysine methyl ester decreased greatly by substitution of the hydrogen of ϵ-NH_2 with methyl group. This was attributed to the lack of H-bondable atoms at the end of the side chain. The results reported in the present paper are obviously at variance with these findings. We cannot offer any definitive explanation for this difference at present. However, it is quite possible that mechanism of peptide cleavage might require different structural requirements from that of the esterase activity of tyrpsin. It is of interest to note that not only trypsin but also α-chymotrypsin hydrolyzed equally the methylated and native ribonuclease (Figure 4). Since α-chymotrypsin hydrolyzes the peptide bonds whose carbonyl group is donated by aromatic amino acids, the result in Figure 4 indicates that the tertiary structure of ribonuclease did not undergo any significant change by reductive methylation.

The results presented in this paper dispel one of the hypothesis on protein methylation that methylation might be a device to protect the protein from the hydrolysis of intracellular proteolytic enzymes. Obviously, a possibility exists that some hither-to-unknown protease will hydrolyze at a different rate when the protein is methylated.

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