# A Comparison of Kinetic Parameters of Polypeptide Substrates for Protein Methylase II†

M. Jamaluddin,‡ S. Kim,* and W. K. Paik

ABSTRACT: Kinetic properties of protein methylase II (*S*-adenosylmethionine:protein *O*-methyltransferase, EC 2.1.1.24) which methylates (esterifies) the free carboxyl side chains of amino acids in proteins was studied using various polypeptides as methyl acceptor substrates. Bovine pancreatic ribonuclease, a model substrate for the enzyme, was subjected to specific cleavage by cyanogen bromide, trypsin, and performic acid oxidation. Several polypeptide fragments derived were then separated by molecular sieve chromatography on a column of Sephadex G-25. The method was found to be very simple and gave good yields. $K_m$ values for these polypeptides as well as a few other protein substrates were determined. While $K_m$ values for the isolated peptides range generally between 4.8 and $0.7 \times 10^{-3}$ M, those of native bovine pancreatic ribonuclease, luteinizing hormone, and follicle-stimulating hormone were determined to be $4.0 \times 10^{-4}$, $5.0 \times 10^{-5}$, and $0.77 \times 10^{-5}$, respectively. Sites of enzymatic methylation of the native ribonuclease were also investigated. Although polypeptides derived from the C-terminal and N-terminal regions of the molecule were found to accept methyl groups, they were unable to undergo enzymatic methylation when native molecule was used as the substrate indicating that within the native ribonuclease these regions are in a conformation which do not allow them to be methylated by protein methylase II under the present assay conditions.

Protein methylase II (*S*-adenosylmethionine:protein *O*-methyltransferase, EC 2.1.1.24) catalyzes the methyl esterification of carboxyl side chains of proteins (Liss et al., 1969; Kim and Paik, 1970; Diliberto and Axelrod, 1974). The enzyme is widely distributed in various mammalian tissues (Paik and Kim, 1971). The properties of the enzyme isolated from various sources indicate that it can catalyze the transfer of methyl group from *S*-adenosyl-L-methionine to a wide variety of purified proteins in vitro (Kim and Paik, 1970; Kim and Paik, 1971a; Kim, 1974).

Earlier studies from this laboratory showed that tryptic or tryptic-chymotryptic cleavage of performic acid oxidized bovine pancreatic ribonuclease did not much affect its capacity to function as a substrate for the enzyme (Kim and Paik, 1971b), suggesting that molecules smaller than the total sequence of peptides of the protein could function as good substrates for the enzyme. On the other hand, pronase digestion of the protein reduced the methyl acceptability markedly and glutathione, dipeptides of dicarboxylic acids, and free amino acids could not serve as substrate for the enzyme (Kim and Paik, 1971a). These results, therefore, indicate that peptides are active as substrate but there must be some limiting size and sequence in which a peptide will no longer be an active substrate for protein methylase II. We felt it important to investigate various isolated peptides as substrates for protein methylase II and compare their activity with those of protein substrate. We have not investigated various oligo- and polypeptides derived from bovine pancreatic ribonuclease and compared their activity with those of the intact ribonuclease molecule and two protein hormones from anterior pituitary gland reported to be good substrates for protein carboxymethylase[1] from bovine pituitary gland (Diliberto and Axelrod, 1974). The results constitute the subject matter of the present communication.

## Materials and Methods

*Materials.* Five times crystallized bovine pancreatic ribonuclease, S peptide, S protein, trypsin, pepsin, and carboxypeptidase A-DFP were purchased from Sigma Chemical Co. The C-terminal pentapeptide of bovine pancreatic ribonuclease was purchased from Schwarz/Mann. Luteinizing hormone (sheep) and follicle-stimulating hormone (porcine) were from Nutritional Biochemical Corporation. *S*-Adenosyl-L-[*methyl*-$^{14}$C]methionine (specific activity, 58 mCi/mmol) was obtained from New England Nuclear Corporation. Other chemicals used were obtained from various commercial sources and were of the highest purity available. Protein methylase II purified from calf thymus (Kim, 1973) kept stabilized in 50% (v/v) glycerol was used.

*Enzyme Assay.* The incubation conditions for the protein methylase II reaction were essentially the same as previously described (Kim and Paik, 1970). The methyl transferred to peptide or protein substrate was assayed by a variation of the method of Axelrod et al. (Axelrod and Daly, 1965). The assay system contains in a total volume of 0.5 ml: 0.1 ml of citrate–phosphate buffer, pH 6 (contains 31 μmol of disodium phosphate and 9.4 μmol of citric acid; $\mu = 0.486$) (Kim and Paik, 1970); 1 μmol of EDTA;[2] 6 μmol of 2-mercaptoethanol; 5 nmol of *S*-adenosyl-L-[*methyl*-$^{14}$C]methionine ($4.5 \times 10^5$ cpm); substrate; 2 μg of enzyme. The incubation was for 10 min at 37 °C. All components of the reaction mixture except *S*-adenosyl-L-methionine were mixed together in ice and preincubated at 37 °C for 4 min before initiating the reaction with

† From the Fels Research Institute and the Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pennsylvania 19140. *Received November 5, 1975.* This work was supported by Research Grant AM09603 from the National Institute of Arthritis and Metabolic Diseases, CA10439 and CA12226 from the National Cancer Institute, and GM20594 from the National Institute of General Medical Sciences.

‡ Present address: Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55101.

[1] Protein carboxymethylase and protein methylase II are identical enzymes (Kim, 1973; Diliberto and Axelrod, 1974).

[2] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane.

*S*-adenosyl-L-methionine. The reaction was stopped by the additon of 1 ml of 0.125 M sodium borate, pH 10.0, and kept at the incubation temperature for 1 min and then transferred to ice. Five milliliters of 3-methyl-1-butanol was added to the tube and left in ice for at least 5 min and then mixed vigorously for 15 s in a Vortex mixer. The layers were separated by centrifugation in a clinical centrifuge for 2 min. Two milliliters of the organic layer was mixed with 10 ml of Scintiverse (Fisher) and the radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer. A blank to which the enzyme was added after arresting the reaction was also run simultaneously and the difference between the counts of the two tubes was taken as a measure of the amount of methanol formed as a result of the alkaline hydrolysis of the protein or peptide methyl ester. Enzyme activities are expressed as picomoles of *methyl*-$^{14}$C transferred per min per mg of enzyme protein.

*Preparation of Ribonuclease C Peptide and C Protein.* Bovine pancreatic ribonuclease C peptide (residues 1–13) and C protein (residues 14–124) were prepared by cyanogen bromide cleavage of ribonuclease (Gross and Witkop, 1962). Cyanogen bromide (408 mg) was dissolved in 0.1 N HCl and a solution of 548 mg of ribonuclease in 0.1 N HCl was added to it (total volume 55 ml) and kept stirred at room temperature (25 °C) in a stoppered flask for 30 h. The reaction mixture was lyophilized and dissolved in the minimum volume of 10% (v/v) formic acid and applied to a column of Sephadex G-25 (2.0 × 135 cm) and eluted with the same solvent at a flow rate of 20 ml/h. Fractions (2 ml) were collected. Fractions 78–90 (C protein, located by absorbance at 280 nm) and 95–100 (C peptide, located by ninhydrin reaction) were pooled together and lyophilized. The C peptide was further purified by re-chromatography on the same column and lyophilization. Automatic amino acid analysis showed it to correspond to the N-terminal tridecapeptide of pancreatic ribonuclease except for the presence of homoserine instead of methionine.

*Preparation of Performic Acid Peptide from C Protein.* Three hundred milligrams of C protein prepared as described above was dissolved in 10 ml of formic acid and cooled in ice. One milliliter of $H_2O_2$ (30%) was added and the reaction mixture was kept for 3 h with occasional shaking. One hundred milliliters of water was added to the reaction flask and the contents lyophilized. The lyophilized material was dissolved in 3 ml of 10% formic acid and applied to a column of Sephadex G-25 (2.0 × 135 cm) and eluted as described above. The positions of the peptide peaks were followed by measuring the absorbance at 280 nm. The second peak emerging between fractions 103 and 116 contained a peptide whose amino acid composition corresponded to the sequence of amino acid residues from 14 to 29 of pancreatic ribonuclease except for the presence of homoserine instead of methionine and cysteic acid instead of cystine.

*Tryptic Cleavage of C Peptide.* C peptide (22 mg) was treated with 0.4 mg of trypsin in a total column of 1.5 ml of 0.2 M sodium borate buffer, pH 7.8 at 37 °C for 1 h. The reaction mixture was applied directly to a column of Sephadex G-25 (3.0 × 148 cm) and the column was eluted with 10% formic acid. Fractions (2 ml) were collected and peptide peaks were located by quantitative ninhydrin color reaction. Fractions 111–131 were pooled together and lyophilized and the peptide was purified by re-chromatography on the same column and lyophilization. Quantitative amino acid analysis showed that the peptide has amino acid composition corresponding to the N-terminal heptapeptide of bovine pancreatic ribonuclease.

*Estimation of Kinetic Parameters.* Maximum velocity ($V_{max}$) and Michaelis constants ($K_m$) were determined graphically by the method of Lineweaver and Burk (Lineweaver and Burk, 1934).

*Estimation of Proteins.* Protein concentration in enzyme solutions was estimated by the method of Lowry et al. (Lowry et al., 1951) using bovine albumin dried over silica gel as the standard.

*Automatic Amino Acid Analysis.* Automatic amino acid analysis was performed by the accelerated amino acid analysis as described by Piez and Morris (Piez and Morris, 1960) and Hamilton (Hamilton, 1963), using a column (0.9 × 56 cm) of Aminex A-5 resin (Bio-Rad), in a Perkin-Elmer KLA-3B analyzer.

*Enzymatic Methylation of Ribonuclease.* Ribonuclease (25 mg) was incubated in the standard incubation mixture (Kim and Paik, 1970) with excess amount of enzyme (10 μg) for 1 h. Four such identical reaction mixtures were pooled together and the methylated ribonuclease was separated from the unreacted *S*-adenosylmethionine by gel filtration on a Sephadex G-25 column (2.0 × 135 cm).

*Peptic Digestion of Enzymatically Methylated Bovine Pancreatic Ribonuclease.* Peptic digestion of enzymatically methylated ribonuclease was performed essentially the same as described by Fugioka and Scheraga (Fugioka and Scheraga, 1965). The methylated ribonuclease (12.3 mg) was dissolved in 1 ml of dilute HCl and an aqueous solution of pepsin (45 μg) was added. Final pH of the reaction was 2.2. Hydrolysis was conducted at room tempeature for a period of 20 min. The reaction mixture was then applied to a column of Sephadex G-25 (2.0 × 135 cm) equilibrated with 10% formic acid (v/v) and eluted with the same solvent. Fractions of 2 ml were collected and the radioactivity in the fractions was determined by mixing 0.5-ml aliquots with 10 ml of Scintiverse (Fisher) and counting in a Packard liquid scintillation spectrometer. Elution position of peptides was determined by quantitative ninhydrin color.

## Results

*Comparative Substrate Activities and Kinetic Parameters for Protein Methylase II.* The substrate activity of various peptides and proteins derived from bovine pancreatic ribonuclease as well as that of the intact ribonuclease toward protein methylase II is presented in Table I. All peptides tested were active as substrates to varying degrees. The N-terminal S peptide was 92% as active as the intact ribonuclease molecule. The C-terminal pentapeptide, Phe-Asp-Ala-Ser-Val, showed 44% of the substrate activity of ribonuclease while the N-terminal heptapeptide, Lys-Glu-Thr-Ala-Ala-Ala-Lys, was only 13% as effective. Removal of the N-terminal S peptide appears to have little influence on the substrate activity since the S protein has about the the same activity as the whole molecule of ribonuclease. These results suggested that a better comparison of the relative effectiveness of the various peptides and proteins could be made on the basis of their kinetic parameters, although the precise position of methylated site in various polypeptides substrates are not known. Table II gives the kinetic parameters of the various peptides and proteins derived from ribonuclease as well as those of two protein hormones. It is seen that peptide substrates derived from bovine pancreatic ribonuclease generally have Michaelis constants which are of the same order of magnitude and at least one or two orders of magnitude higher than those of protein substrates.

*Pentapeptide (Phe-Asp-Ala-Ser-Val) as Substrate for Protein Methylase II.* A relatively good substrate for the calf thymus enzyme is the small pentapeptide derived from the C

TABLE I: Substrate Activity and Some Properties of Peptides and Proteins Derived from Bovine Pancreatic Ribonuclease Toward Calf Thymus Protein Methylase II.[a]

| Substrate | Sp Act.[b] | Amino Acid Residues | N Terminal | C Terminal | Asp | Glu |
|---|---|---|---|---|---|---|
| Native ribonuclease | 3400 | 124 | Lys | Val | 5 | 5 |
| C protein[c] | 4240 | 111 | Asp | Val | 5 | 3 |
| S protein[c] | 3460 | 104 | Ser | Val | 4 | 3 |
| S peptide (N-terminal peptide) | 3080 | 20 | Lys | Ala | 1 | 2 |
| Performic acid peptide | 2780 | 16 | Asp | Hse | 1 | 0 |
| C peptide (N-terminal peptide) | 806 | 13 | Lys | Hse | 0 | 2 |
| Heptapeptide (N-terminal peptide) | 450 | 7 (Lys-Glu-Thr-Ala-Ala-Ala-Lys) | Lys | Lys | 0 | 1 |
| Pentapeptide (C-terminal peptide) | 1480 | 5 (Phe-Asp-Ala-Ser-Val) | Phe | Val | 1 | 0 |

[a] Assay conditions were standard. All substrates were dissolved in 0.1 M tris(hydroxymethyl)aminomethane hydrochloride buffer, pH 8.0, and adjusted to pH 6.0 by 0.3 N HCl. Methyl acceptor substrate (0.65 μmol) was present in the assay system. [b] Specific activity is expressed as pmol of methyl transferred per min per mg of enzyme protein. [c] Precipitates were noted during the incubation.

TABLE II: Kinetic Parameters of Various Peptides and Proteins for Calf Thymus Protein Methylase II.[a]

| Substrate Tested | $K_m$ (mol/l.) | $V_{max}$ (pmol $min^{-1}$ $mg^{-1}$ Protein) |
|---|---|---|
| Heptapeptide | $3.6 \times 10^{-3}$ | 1830 |
| C peptide | $4.8 \times 10^{-3}$ | 4000 |
| S peptide | $2.0 \times 10^{-3}$ | 8400 |
| Pentapeptide | $3.2 \times 10^{-3}$ | 5600 |
| Performic acid peptide | $0.71 \times 10^{-3}$ | 4870 |
| Bovine pancreatic ribonuclease | $4.0 \times 10^{-4}$ | 4110 |
| C protein | $2.5 \times 10^{-5}$ | 5100 |
| Luteinizing hormone (sheep) | $5.0 \times 10^{-5}$ | 6110 |
| Follicle-stimulating hormone (porcine) | $0.77 \times 10^{-5}$ | 7150 |

[a] Assay conditions were standard. $K_m$ and $V_{max}$ values were obtained from Lineweaver-Burk plots of initial velocity data obtained with varying concentrations of the substrates.

TABLE III: Effect of Treating Pentapeptide with Carboxypeptidase A on Its Capacity to Act as a Substrate for Protein Methylase II.[a]

| | Mol of Amino Acid Removed/mol of Peptide | | | |
|---|---|---|---|---|
| Period of Incubation (min) | Val | Ser | Ala | Act.[b] (%) |
| 0 | 0 | 0 | 0 | 100 |
| 5 | 0.90 | 0.02 | 0 | 45 |
| 10 | 1.00 | 0.04 | 0 | 37 |
| 15 | 1.01 | 0.08 | 0 | 32 |
| 30 | 1.03 | 0.19 | 0 | 38 |
| 60 | 0.97 | 0.38 | 0 | |
| 90 | 1.00[c] | 0.48 | 0.004 | |

[a] Six milligrams of pentapeptide was dissolved in 0.9 ml of 0.05 M Tris-HCl buffer (pH 9.0) and 1 mg of carboxypeptidase A was added; the mixture was mixed well and incubated at room temperature. Aliquots (0.1 ml) were withdrawn at indicated intervals of time into 0.05 ml of 0.3 N HCl, and kept overnight at room temperature. An aliquot (0.025 ml) was withdrawn for quantitative amino acid analysis and the rest was assayed for substrate activity. A reaction mixture without pentapeptide substrate taken through the same treatment served as control at the indicated intervals of time. [b] 100% activity represents specific activity of 1100. [c] Taken as standard.

terminus of pancreatic ribonuclease. This afforded an opportunity to test the effect of removing the amino acid residues sequentially from C terminus on its ability to function as a substrate. The effect of treating the pentapeptide with carboxypeptidase A is shown in Table III. After 5 min there was a 45% fall in activity followed by a 32–38% decrease in activity which remained at the end of 30-min digestion at which time the C-terminal valine is completely cleaved off (Table III). These results indicate that enzymatic methylation of the pentapeptide results in the methyl esterification of the valine carboxyl residue which accounts for about 70% of the total radioactivity incorporated into the molecule.

*Site Specificity of Methylation of Pancreatic Ribonuclease by Protein Methylase II.* The fact that the C-terminal pentapeptide derived from pancreatic ribonuclease acted as a methyl acceptor site prompted us to examine whether the C terminus of the native ribonuclease could be a methyl acceptor site. Ribonuclease was enzymatically methylated and separated from the unreacted *S*-adenosylmethionine by gel filtration in a Sephadex G-25 column and lyophilization. The methylated ribonuclease was then cleaved by pepsin. Anfinsen (Anfinsen, 1956) and Fugioka and Scheraga (1965) showed that the early event in the cleavage of ribonuclease by pepsin was the removal of the C-terminal tetrapeptide. The elution pattern of radioactivity after treatment of methylated ribonuclease by pepsin is shown in Figure 1. The figure shows that there was no measurable radioactivity in the elution position expected for the peptide, showing that the C terminus of the ribonuclease molecule is not a significant methylation site. The ninhydrin peak of tetrapeptide is clearly shown.

When the methylated ribonuclease was cleaved with CNBr and separated into C protein and C peptide as shown in Figure 2, no radioactivity was observed in the elution position of the C peptide, suggesting that the N-terminal region of ribonuclease also is not a significant methyl acceptor site. The C protein peak was pooled together and lyophilized and oxidized with performic acid as described in the Materials and Methods section. The performic acid oxidized material was chromatographed on a Sephadex G-25 column and the results are shown in Figure 3. The figure reveals that the peptide region (residues 14–29) of the ribonuclease molecule also does not seem to accept the methyl group to any significant extent. Although the recovery of radioactivity after the treatment described above varied from 80 to 85%, it is unlikely that the loss selectively occurred at both the C- and N-terminal regions of the molecule, because acidic conditions were used in all of the treatments. Riehm et al. (1965) found that extended exposure

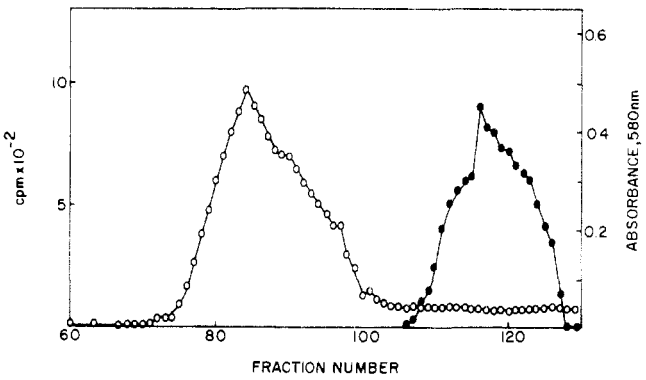


FIGURE 1: Sephadex G-25 column chromatographic pattern of pepsin digest of enzymatically methylated bovine pancreatic ribonuclease. Enzymatically methylated ribonuclease (12.3 mg) containing 15 000 cpm in 1 ml was applied to a column of Sephadex G-25 (2.0 × 135 cm) and eluted with 10% formic acid (v/v) at a flow rate of 60 ml per h. Fractions (2 ml) were collected automatically. One-half-milliliter aliquots of the fractions were counted for the radioactivity. (○) Counts per minute; (●) ninhydrin color.

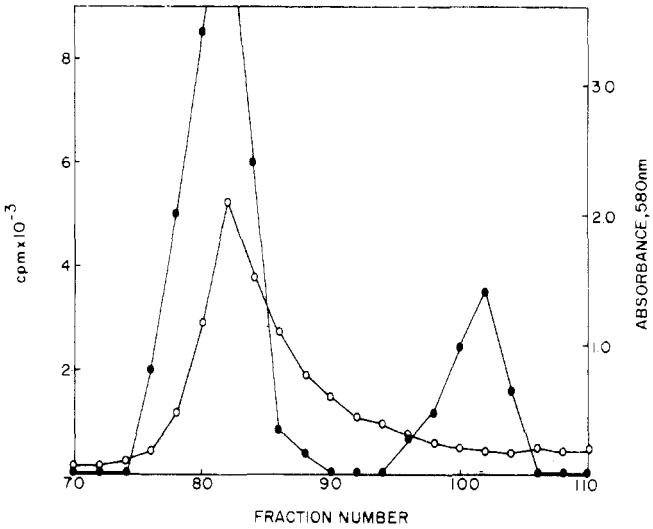


FIGURE 2: Sephadex column chromatographic pattern of CNBr-cleaved enzymatically methylated pancreatic ribonuclease. Enzymatically methylated pancreatic ribonuclease (50 mg; 65 000 cpm) was dissolved in 4 ml of 0.1 N HCl and added to 50 mg of CNBr in 0.4 ml of 0.1 N HCl. The reaction mixture was kept stirred in a stoppered vessel for 24 h at room temperature and lyophilized. The residue was dissolved in 1 ml of 10% formic acid and applied to a column of Sephadex G-25 and eluted at a flow rate of 20 ml per h. Fractions (2 ml) were collected. Aliquots (0.2 ml) were used to measure radioactivity, and 0.1 ml for ninhydrin color development. (○) Counts per minute; (●) ninhydrin color.

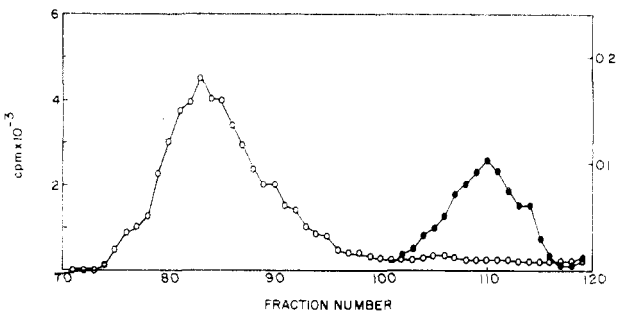


FIGURE 3: Sephadex column chromatographic pattern of oxidized C protein obtained from Figure 2. C protein (40 mg) obtained by lyophilizing the pooled radioactive peak of Figure 2 (52 000 cpm) was dissolved in 2.5 ml of formic acid and 1.5 ml of performic acid prepared by mixing together 4.75 ml of formic acid and 0.25 ml of $H_2O_2$ and keeping it in ice for 2 h. The reaction mixture was kept in ice for 2.5 h and lyophilized. The lyophilized material was dissolved in 1 ml of 10% formic acid and applied to a column of Sephadex G-25 (2.0 × 135 cm) and eluted with the same solvent at a flow rate of 60 ml per h. Fractions (2 ml) were collected. Aliquots (0.2 ml) were mixed with 10 ml of Scintiverse and the radioactivity was counted. (○) Counts per minute; (●) absorbance at 280 nm.

of methylated ribonuclease to acidic conditions and performic acid oxidation does not lead to any significant ester hydrolysis. Riehm and Scheraga (1965) did not observed ester hydrolysis on prolonged peptic treatment of the product of reaction between ribonuclease and diazoacetoglycinamide. Therefore, it seems reasonable to conclude that the methyl acceptor region in the native ribonuclease molecule is somewhere in the middle of the molecule between residues 31 and 120.

## Discussion

The results of the present paper suggest that protein methylase II can methylate diverse peptides and proteins. Proteins show the lowest Michaelis constants (Table II) suggesting that proteins might be the preferred substrates. In native bovine pancreatic ribonuclease, at least, the methylation appears to occur at some internal residues away from the N and C termini. This might be because of the unavailability of the residues at the termini for methylation due to the tertiary structure of the ribonuclease molecule in solution of acidic pH, because in the case of peptide substrates it appears that methylation occurs at the C termini as well. Pancreatic ribonuclease has 11 carboxyl residues, 5 each of glutamic and aspartic acid carboxyls. However, residues 14, 38, and 83 are "buried" and not available for chemical methylation even on prolonged reaction (Riehm et al., 1965). It is reasonable to assume that these are also not available for enzymatic methylation. The results of the present paper suggest that Asp-121 and Val-124 are not major methyl acceptor site in native ribonuclease since no radioactive methyl group was incorporated into the N-terminal tetrapeptide (Figure 1). Therefore among the aspartic acid residues Asp-53 might be a methyl acceptor site. Riehm and Scheraga (1965) and Acharya and Vithayathil (1975) showed that Asp-53 of pancreatic ribonuclease is an exceptionally reactive carboxyl group. The results of Acharya and Vithayathil also indicated that Glu-49 is a fast-reacting group and that the carboxyl end undergoes reaction only after Asp-53 and Glu-49 have reacted. The present results suggest that Glu-2 and Glu-9 do not undergo methylation to any significant extent since the first 13 amino acids in the native ribonuclease did not incorporate radioactive methyl groups (Figure 2). Thus, Glu-86 and Glu-111 are left as the other possible sites of enzymic methylation. Since Glu-111 is in a region near two bulky disulfide bridges (residues 65–72 and 58–110) which are close together (Kartha et al., 1967), it might not be a good methyl acceptor site, while Glu-86 being in a region projecting into the medium appears to be a good candidate for undergoing methylation.

The substrate activities of the various peptides (derived from pancreatic ribonuclease) tested depend on the length of the peptide and sequence. The heptapeptide, Lys-Glu-Thr-Ala-Ala-Ala-Lys, is only about one-third as active as the pentapeptide, Phe-Asp-Ala-Ser-Val, suggesting a certain degree of amino acid sequence specificity. Increasing the length of the peptide while keeping the amino terminal end the same resulted in a progressive increase in the substrate activity as revealed in the doubling of the maximum velocity in going from the heptapeptide to the tridecapeptide and then from the tridecapeptide to the peptide of 20 amino acids (second and third

column of Table II), though the Michaelis constants were not significantly affected. This increase of maximum velocity may be due to the increasing number of methylatable sites present in the peptides since it correlates well with the increased number of carboxyl groups available for methylation as the peptide chain lengthens (see last two columns of Table I). In fact all the peptides tested had Michaelis constants of the same order of magnitude but had widely different maximum velocities.

Kinetic studies carried out earlier (Jamaluddin et al., 1975) showed a rapid equilibrium random mechanism for the calf thymus protein methylase II catalyzed reaction. Nonvariability of Michaelis constants for the various peptides could then be explained on the basis that they have equal affinities for the free enzyme. Although shorter peptides have similar affinities for the enzyme, the variation in $V_{max}$ depends on the number and location of the carboxyl groups available for methylation. Thus in the case of small peptides, which lack a tertiary structure, the previous mentioned factors are the major influence on the rate of breakdown of the ternary enzyme–substrate complex in the formation of the products. However, in the case of protein, the variability of Michaelis constant for the protein substrates is to be expected since their affinity for the free enzyme seems to be dependent mainly on protein conformation. The fact that the protein obtained by removing the N-terminal tridecapeptide from pancreatic ribonuclease (C protein) has a Michaelis constant an order of magnitude lower than that of the intact ribonuclease may be explained in this way. The variation of the maximum velocities reflects the variability of the rates of interconversion of the enzyme–protein–*S*-adenosylmethionine and enzyme–protein–ester–*S*-adenosylhomocysteine complexes, as well as the variation of the number and kind of carboxyl groups of the protein that are available for methylation.

Among the protein substrates tested, porcine follicle-stimulating hormone appears to have the highest affinity for the calf thymus protein methylase II ($K_m$ = 7.7 $\mu$M). Diliberto and Axelrod, 1974) reported that several anterior pituitary hormones are good substrates for a protein carboxymethylase of bovine pituitary. Our results show that two of them are also good substrates for the calf thymus enzyme. Thus there does not seem to be any tissue specific substrate specificity for the enzyme.

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References

Acharya, A. S., and Vithayathil, P. J. (1975), *Int. J. Pept. Protein Res. 7,* 207–219.
Anfinsen, C. B. (1956), *J. Biol. Chem. 221,* 405–411.
Axelrod, J., and Daly, J. (1965), *Science 150,* 892–893.
Diliberto, E. J., Jr., and Axelrod, J. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1701–1704.
Fugioka, H., and Scheraga, H. A. (1965), *Biochemistry 4,* 2197–2206.
Gross, E., and Witkop, B. (1962), *J. Biol. Chem. 237,* 1856–1860.
Hamilton, P. B. (1963), *Anal. Chem. 35,* 2055–2064.
Jamaluddin, M., Kim, S., and Paik, W. K. (1975), *Biochemistry 14,* 694–698.
Kartha, G., Bello, J., and Harker, D. (1967), *Nature (London), 213,* 862–865.
Kim, S. (1973), *Arch. Biochem. Biophys. 157,* 476–484.
Kim, S. (1974), *Arch. Biochem. Biophys. 161,* 652–657.
Kim, S., and Paik, W. K. (1970), *J. Biol. Chem. 245,* 1806–1813.
Kim, S., and Paik, W. K. (1971a), *Anal. Biochem. 42,* 255–261.
Kim, S., and Paik, W. K. (1971b), *Biochemistry 10,* 3141–3145.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56,* 658–666.
Liss, M., Maxam, M., and Cuprak, L. J. (1969), *J. Biol. Chem. 244,* 1617–1622.
Lowry, O. H., Rosebrough, N. J. Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265–275.
Paik, W K., and Kim, S. (1971), *Science 174,* 114–119.
Peiz, K. A., and Morris, L. (1960), *Anal. Biochem. 1,* 187–201.
Riehm, J. P., Broomfield, C. A., and Scheraga, H. A. (1965), *Biochemistry 4,* 760–771.
Riehm, J. P., and Scheraga, H. A. (1965), *Biochemistry 4,* 772–782.