S-Adenosylmethionine:Protein-Arginine Methyltransferase. Purification and Mechanism of the Enzyme†

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ABSTRACT: Protein methylase I (*S*-adenosylmethionine: protein-arginine methyltransferase, EC 2.1.1.23) has been purified from calf brain approximately 120-fold with a 14% yield. The final preparation is completely free of any other protein-specific methyltransferases and endogenous substrate protein. The enzyme has an optimum pH of 7.2 and p*I* value of 5.1. The K_m values for *S*-adenosyl-L-methionine, histone H4, and an ancephalitogenic basic protein are 7.6×10^{-6}, 2.5×10^{-5}, and 7.1×10^{-5} M, respectively, and the K_i value for *S*-adenosyl-L-homocysteine is 2.62×10^{-6} M. The enzyme is highly specific for the arginine residues of protein, and the end products after hydrolysis of the methylated protein are N^G,N^G-di(asymmetric), N^G,N'^G-di(symmetric), and N^G-monomethylarginine. The ratio of [^{14}C]methyl incorporation into these derivatives by enzyme preparation at varying stages of purification remains unchanged at 40:5:55, strongly indicating that a single enzyme is involved in the synthesis of the three arginine derivatives. The kinetic mechanism of the protein methylase I reaction was studied with the purified enzyme. Initial velocity patterns converging at a point on the extended axis of abscissas were obtained with either histone H4 or *S*-adenosyl-L-methionine as the varied substrate. Product inhibition by *S*-adenosyl-L-homocysteine with *S*-adenosyl-L-methionine as the varied substrate was competitive regardless of whether or not the enzyme was saturated with histone H4. On the other hand, when histone H4 is the variable substrate, noncompetitive inhibition was obtained with *S*-adenosyl-L-homocysteine under conditions where the enzyme is not saturated with the other substrate, *S*-adenosyl-L-methionine. These results suggest that the mechanism of the protein methylase I reaction is a Sequential Ordered Bi Bi mechanism with *S*-adenosyl-L-methionine as the first substrate, histone H4 as the second substrate, methylated histone H4 as the first product, and *S*-adenosyl-L-homocysteine as the second product released.

An enzyme which methylates the guanidino group of arginine residues of protein using *S*-adenosyl-L-methionine as methyl donor has been designated as protein methylase I (*S*-adenosylmethionine:protein-arginine methyltransferase, EC 2.1.1.23). Among the enzymatic products of this reaction which thus far have been identified are N^G-mono-, N^G,N^G-di-, and N^G,N'^G-dimethylarginine[1] (Paik and Kim, 1968; Kakimoto and Akazawa, 1970). The enzyme is widely distributed in various organs of the rat, being especially rich in testis, brain, and thymus (Paik and Kim, 1968, 1971). Although the enzyme activity is found predominantly in the cytosolic fraction of the cell, some activity has also been reported in the nucleus (Gallwitz, 1971).

The level of protein methylase I activity was found to closely parallel the rate of cell proliferation. Elevated activity was observed in fetal tissues (Paik et al., 1972b), continuously dividing HeLa S-3 cell culture (Borun et al., 1972), regenerating adult rat liver (Lee and Paik, 1972), and in fast-growing poorly differentiated hepatomas (Paik et al., 1972a, 1975). In the fast-growing Novikoff hepatoma, the enzyme activity increased approximately ninefold (Paik et al., 1975). Unlike protein methylase III (*S*-adenosylmethionine:protein-lysine methyltransferase, EC 2.1.1.43) which methylates the ϵ-amino group of lysine residues of histones, the increase of protein methylase I activity preceded the peaks of DNA and RNA synthesis in continuously dividing HeLa S-3 cell culture (Borun et al., 1972), suggesting that the function of arginine methylation is different from that of lysine methylation.

In the present paper, protein methylase I has been purified 120-fold from calf brain and the properties of the enzyme as well as the kinetic mechanism of the enzyme reaction have been investigated.

Materials and Methods

Materials. Fresh calf brain was obtained from a local abattoir and was frozen until use. *S*-Adenosyl-L-[*methyl*-^{14}C]methionine (specific activity, 60 mCi/mmol) was purchased from New England Nuclear Corp. (Boston). Histone type II-A (crude), *S*-adenosyl-L-homocysteine, calcium phosphate gel, and other proteins used in the experiments of Table II were obtained from Sigma Chemical Co. (St. Louis). Cellex-D, Bio-Gel A-1.5m, and Bio-Gel A-15m were from Bio-Rad Laboratories. Glycine-rich, arginine-rich histone (H4 histone; F_{2a1}; GAR) used in the kinetic studies was the gift of Dr. H. Busch, Baylor University, Texas (Ogawa et al., 1969). Other chemicals were obtained from various local sources and were of analytical grade. Finally, experimental allergic encephalitogenic basic protein (AI basic protein) was purified from calf brain according to the method published (Eylar et al., 1975), and oxidized pancreatic ribonuclease was prepared by the method described (Hirs, 1956).

Enzyme Assay. A detailed description of the enzyme assay has been described previously (Paik and Kim, 1968). Briefly, 0.1 ml of 0.5 M phosphate buffer (pH 7.2), 0.1 ml of *S*-adenosyl-L-[*methyl*-^{14}C]methionine (10 nmol/0.1 ml; 80–100

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[1] N^G refers to the nitrogen of the guanidino group of arginine, and N^G,N^G-dimethylarginine is dimethyl substituted on the same nitrogen atom in the guanidino group. Thus, this compound is also called asymmetric dimethylarginine. In the same terminology, N^G,N'^G-dimethylarginine is dimethyl substituted at two different nitrogen atoms and, therefore, called symmetric dimethylarginine. AMe represents *S*-adenosyl-L-methionine.

cpm/pmol), 0.1 ml of protein substrate (routinely, 3 mg each of histone type II-A, AI protein, and histone H4 for the kinetic study), and 0.1 ml of enzyme preparation in a 0.5 ml total volume were incubated at 37 °C for 10 min. An enzyme preparation heated at 100 °C for 5 min served as a blank. The reaction was terminated by addition of 0.5 ml of 30% trichloroacetic acid, and the acid-soluble fraction, nucleic acids, and phospholipid were removed by the method described previously (Paik and Kim, 1968). When the reaction mixture was analyzed at this stage, particularly with crude enzyme preparation, there was some radioactivity incorporated into the substrate protein which became volatile on acid-hydrolysis. This radioactivity is due to the action of contaminating protein methylase II (*S*-adenosylmethionine:protein-carboxyl methyltransferase, EC 2.1.1.24). Therefore, 0.5 ml of 0.5 M phosphate buffer at pH 8.0 was added to the above trichloroacetic acid treated sample and the mixture was heated at 60 °C for 5 min. The methyl ester produced by the action of protein methylase II is extremely unstable on alkaline hydrolysis (Kim and Paik, 1970). About 3 ml of 15% trichloroacetic acid was added into the above mixture, and the precipitate was collected by centrifugation. The precipitate was washed once with 95% ethanol and was transferred quantitatively into a scintillation vial which contained 10 ml of scintillation solution. The radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer with 85% efficiency.

Amino Acid Analysis. Proteins were hydrolyzed in 6 N HCl at 110 °C in vacuo for 48 h, and HCl was removed by repeated distillation under reduced pressure by means of a flash evaporator. The amino acid analysis was carried out by the method described by Paik and Kim (1975a); a column (0.9 × 35 cm) of Bio-Rad A-5 resin with a particle size of 13 ± 2 μm was first eluted with 0.38 N sodium citrate buffer, pH 5.84, at 24 °C with a flow rate of 45 ml per h. After 2 h, the buffer was changed to 0.35 N sodium citrate, pH 4.7. Under this condition, all the methylated lysine derivatives were eluted between lysine and histidine. Three methylated arginine derivatives, N^G-mono-, N^G,N^G-di-, and N^G,N'^G-dimethylarginine, were clearly separated from each other. Furthermore, N^G-monomethylarginine was completely resolved from arginine. When studying radioactivity incorporation into each methylated arginine derivative, the radioactivity was monitored by a flow cell of a Packard Tri-Carb Model 2002 liquid scintillation spectrometer with a counting efficiency of approximately 60%.

Isoelectric Focusing. The p*I* value was determined by isoelectric focusing and was carried out by the method described by Vesterberg (1971); the carrier ampholytes used in this investigation were selected to give a pH gradient between 3 and 10 and were used in final concentration of 1% (w/v) to establish the pH gradient. Focusing and separation of the protein were carried out at 4 °C in a special vertical electrolysis column of 110-ml capacity, equipped with a cooling jacket (LKB-Produkter AB, Stockholm-Bromma, Sweden).

The enzyme (approximately 14 mg of protein in 0.5 ml) was mixed with one-third of the carrier ampholyte and this was used as the "less" dense solution. The dense solution contained 50% (w/v) of sucrose and two-thirds of the carrier ampholyte. The central tube surrounding the anode was filled with a 50% sucrose solution containing 0.2 ml of concentrated H_2SO_4. For prevention of contact between the ampholyte and the cathode, 10 ml of 2% (w/v) ethylenediamine was added to the top of the column. After focusing for 30 h with a potential of 500 V, the contents of the column was collected in 1.0-ml fractions. The pH of the fractions was determined for every other fraction, and the amount of protein and enzyme activity in each fraction was measured. The concentration of enzyme protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Enzyme Purification. Step 1. Fresh calf brain obtained from a slaughterhouse was kept frozen until use. The enzyme activity remained unchanged up to 3 months. One hundred grams of frozen brain was thawed and homogenized in 4 volumes of precooled 0.25 M sucrose–1 mM EDTA[2] in a Waring Blendor for 20–30 s at 30 V. This step and the following procedures were carried out at 0–3 °C. The crude homogenate was further homogenized in a Teflon–glass homogenizer at 900 rpm and passed through a double layer of cheesecloth followed by centrifugation at 105 000*g* for 1 h in a Beckman L2 ultracentrifuge. A lipid layer on top of the supernatant was removed by passage through glass wool.

Step 2: First Ammonium Sulfate Precipitate. Per 100 ml of supernatant solution, 31.3 g of crystalline ammonium sulfate (analytical grade) was added slowly under continuous stirring within 15–20 min. The solution was allowed to stand in the cold for at least 30 min after all ammonium sulfate was dissolved. The precipitate was collected by centrifugation at 18 000 rpm (39 000*g*) in a Sorvall Superspeed centrifuge and dissolved in approximately 100 ml of cold water.

Step 3: Calcium Phosphate Gel Treatment. Five volumes of calcium phosphate gel (suspension of 17 mg of solid/ml of water) was added to the enzyme suspension under gentle stirring and allowed to stand for 20 min for complete adsorption. After centrifugation at low speed, the sedimented gel was washed twice by resuspension in a large volume of cold water and centrifugation. For elution of the enzyme, the final gel sediment was resuspended in 60 ml of 0.25 M phosphate buffer (pH 7.2) and allowed to stand for 15–20 min. The eluate was recovered by centrifugation at high speed and the supernatant solution was saved.

Step 4: Second Ammonium Sulfate Precipitation. The eluate was precipitated with crystalline ammonium sulfate (31.3 g per 100 ml) as described above. The precipitate collected by centrifugation was dissolved in about 6 ml of 5 mM phosphate buffer (pH 6.0) containing 3 mg/100 ml dithiothreitol and 10% glycerol. The solution was dialyzed against 2000 ml of water for 4–5 h and subsequently against 2000 ml of 5 mM phosphate buffer (pH 6.0)–3 mg/100 ml of dithiothreitol–10% glycerol for several hours. Initial dialysis against water was necessary to speed up the dialysis process which otherwise takes at least overnight accompanied by a considerable loss in enzyme activity.

Step 5: Cellex-D Chromatography. Cellex-D resin (Bio-Rad) was prepared by the following washes: (1) 0.25 N NaCl–0.25 N NaOH, (2) distilled water, (3) 0.25 N HCl, and (4) water until free of chloride. The resin was then loaded into a 1.2 × 25 cm column and equilibrated with 5 mM phosphate buffer (pH 6.0)–3 mg/100 ml dithiothreitol–10% glycerol. The dialyzed sample obtained in step 4 was applied to the column and eluted with a linear gradient formed by 100 ml of 5 mM phosphate buffer (pH 6.0)–3 mg/100 ml dithiothreitol–10% glycerol and 100 ml of 0.8 M ammonium sulfate in the same solution. Flow rate was maintained at 50–60 ml per h. Protein methylase I activity was recovered between 110 and 130 ml of effluent. Fractions from this range were pooled and concentrated by precipitation with crystalline ammonium sulfate (60% saturation). The solution was stirred for 30 min after all ammonium sulfate was dissolved and centrifuged at 39 000*g*

[2] Abbreviations used: EDTA, ethylenediaminetetraacetic acid.

TABLE I: Purification, Ratio of End Products, and Substrate Efficiency of Histone and AI Protein for Protein Methylase I.[a]

Purification Steps	Purification (fold)	Spec. Act. (pmol min^{-1} (mg of enzyme^{-1})	Total Act. (pmol/min)	End Product (cpm) N^G,N^G-DMA	N^G,N'^G-DMA	N^G-MMA	Substrate Eff. Histone/ AI Protein Ratio
Whole homogenate	1	0.32	2567	1560 (37.7%)	342 (7.63%)	2343 (55.2%)	1.6
Supernatant	4.6	1.49	1713	2260 (41.2%)	330 (6.01%)	2900 (52.8%)	1.7
1st $(NH_4)_2SO_4$ ppt	9.1	2.93	1513	2207 (35.1%)	307 (4.87%)	3780 (60.1%)	
Eluate from $Ca_3(PO_4)_2$ gel	15.7	5.08	1348	2134 (47.8%)	177 (3.96%)	2158 (48.3%)	2.8
2nd $(NH_4)_2SO_4$ ppt	24.2	7.80	1252	2295 (42.9%)	105 (1.96%)	2956 (55.2%)	1.6
Cellex-D	39.3	12.7	818	4757 (46.5%)	450 (4.28%)	5015 (48.9%)	5.0
Bio-Gel A-15m	118.6	38.3	348	3762 (31.4%)	690 (5.10%)	7609 (63.5%)	8.9
Average (%)				40.4 ± 6.0	4.8 ± 1.8	54.9 ± 5.6	

[a] N^G,N^G-DMA, N^G,N'^G-DMA, and N^G-MMA represent N^G,N^G-dimethylarginine (asymmetric), N^G,N'^G-dimethylarginine (symmetric), and N^G-monomethylarginine, respectively.

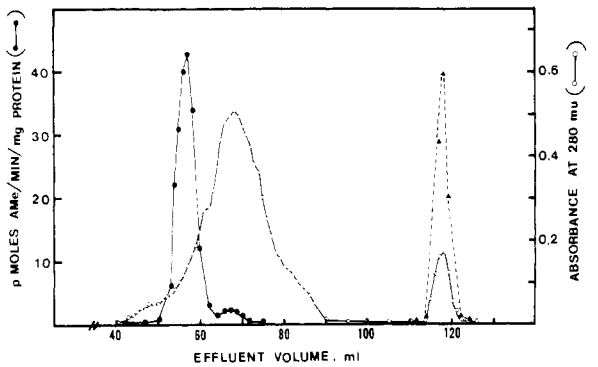

FIGURE 1: Elution profile of protein methylase I on Bio-Gel A-15m. Protein methylase I was eluted from Bio-Gel A-15m column (1.3 × 160 cm) following as described under Materials and Methods and assayed for both protein methylase I and II. (●—●) Protein methylase I activity; (▲ - - - ▲) protein methylase II activity.

for 10 min. The supernatant solution was discarded. The pellet was dissolved in a small volume of 5 mM borate buffer (pH 7.2)–10% glycerol and dialyzed against the same buffer for several hours.

Step 6: Chromatography on Bio-Gel A-15m. A Bio-Gel A-15m column (1.3 × 160 cm) was equilibrated with 5 mM borate buffer (pH 7.2)–10% glycerol for 3–4 days. A portion of the enzyme preparation obtained in step 5 was applied to the column, usually 1–1.5 ml containing 30–50 mg of protein, and eluted with 5 mM borate buffer (pH 7.2)–10% glycerol at a flow rate of 10 ml per h. Ninety-five percent of protein methylase I activity was eluted in a sharp peak just preceding the main protein peak (50–60 ml of effluent; Figure 1). The enzyme preparation was completely free of other protein methylases, especially protein methylase II which was eluted much later from the column (around 120 ml of effluent). The overall purification at this stage was approximately 120-fold with a yield of 14% and specific activity of 38 pmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine used per min per mg of enzyme protein. Pertinent data on the various steps in a typical procedure are summarized in columns 1–4, Table I.

Treatment of Kinetic Data. All initial velocities are expressed as pmol of methyl groups incorporated into histone H4 (F_{2a1}; GAR) per min per 0.5 ml of the reaction mixture. Initial velocity data were first analyzed graphically by plotting the reciprocals of velocities against the reciprocals of substrate concentrations. When these plots were linear, the data were fitted to eq 1 according to the least-square method of Wilkinson (1961) assuming equal variance for the velocities.

$$v = \frac{VA}{K_a + A} \quad (1)$$

where v, V, K_a, and A represent initial velocity, maximum velocity, Michaelis constant, and substrate concentration, respectively. Intercepts ($1/V$) and slopes (K/V) of such fits were plotted graphically against the reciprocals of the concentrations of nonvaried substrate to extract constants (Table III).

Results

Enzyme Purification. As depicted in Table I (columns 1–4), protein methylase I has been purified from calf brain approximately 120-fold with a 14% yield. The final preparation was found to be completely free of protein methylase II and III activities as well as endogenous substrate protein (measured by assaying the enzyme activity without added exogenous substrate protein). However, the preparation is not homogeneous, showing several bands on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. When the enzyme preparation, after Cellex-D treatment (Table I), was applied on Bio-Gel A-1.5m (exclusion limit of 1.5×10^6 according to the chart distributed by Bio-Rad Laboratories), the enzyme activity was found in the void volume without any additional purification (figure not shown). This magnitude of apparent molecular weight of the protein methylase I preparation might be due to the enzyme being complexed with some high-molecular-weight compound such as DNA or RNA. However, pretreatment of the enzyme preparation with either DNase or RNase failed to alter this pattern of elution (figure not shown). The above result, therefore, indicates that the molecular weight of the enzyme exceeds 1.5×10^6. Because of the inherent instability of the enzyme, we did not pursue the question of whether a molecular size of this magnitude is due to protein aggregation.

Effect of pH and Incubation Period. Because of uncertainty of whether a single enzyme is involved in methylation of histone and AI protein in vivo and in vitro, we examined the effect of pH (left panel of Figure 2) and incubation period (right panel of Figure 2) on the enzyme activity, using both histone and AI protein as substrates. It is shown in the figure that histone serves as a much better substrate than AI protein, and that a pH optimum of around 7 is obtained with both substrates. The enzyme activity is directly proportional to the incubation time

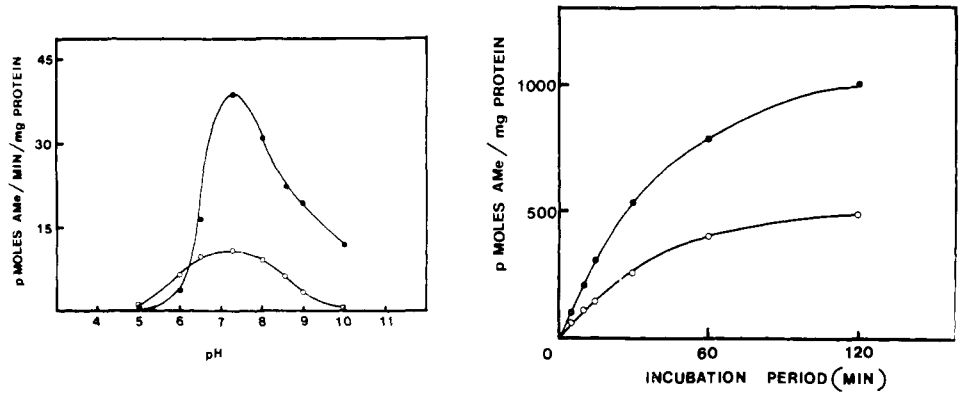

FIGURE 2: Effect of pH and incubation period. (Left) Effect of pH. Citrate-phosphate buffer (1 part of 0.5 M disodium phosphate plus 0.6 part of 0.25 M citric acid) was used in the pH range 4.8-6.0, 0.5 M phosphate buffer for 6.5-8.0, 0.5 M Tris-HCl buffer for 8.5-9.0, and 0.5 M glycine-NaOH buffer for pH 10.0. (●—●) For histone type II-A (3 mg) as the substrate and (○—○) AI basic protein (3 mg). (Right) Effect of incubation period. (●—●) For histone type II-A as the substrate (3 mg) and (○—○) for AI basic protein (3 mg). Assay was performed as described under Materials and Methods.

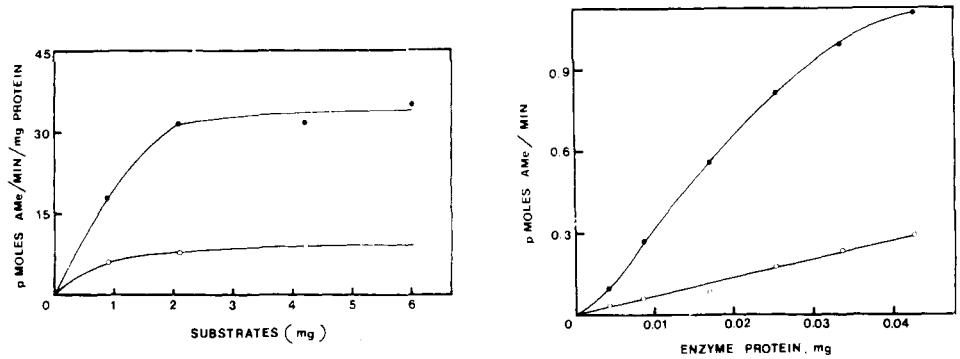

FIGURE 3: Effect of varying the protein substrate and enzyme concentration on the rate of protein methylase I activity. (Left) Various amounts of histone H4 (●—●) or AI basic protein (○—○) were used in the assay. Purified protein methylase I (0.021 mg) was used in the assay procedure described under Materials and Methods. (Right) Effect of enzyme concentration. Histone type II-A (3 mg) (●—●) or AI basic protein (○—○) was assayed with varying amounts of purified enzyme preparation.

up to about 20 min. We have chosen a 10-min incubation period in the routine assay.

Effect of Substrates and Enzyme Concentration. The left panel of Figure 3 illustrates the effect of histone and AI protein concentration on the enzyme activity. It is seen that substrate efficiency of AI protein is only about 30% of that obtained with histone, and that 3 mg of protein is sufficient to saturate the enzyme under this condition. When plotted by the Lineweaver-Burk equation, K_m values of 2.5×10^{-5} and 7.1×10^{-5} M were obtained for histone and AI protein, respectively. Similarly, the K_m value for *S*-adenosyl-L-methionine, which is another substrate for protein methylase I, was 7.6×10^{-6} M (not shown). The right panel of Figure 3 shows the effects of enzyme concentration on the activity. Although the enzyme activity is directly proportional to the amount of enzyme with AI protein as substrate, a slight sigmoidal curve was obtained when histone served as substrate. This result is highly reproducible and a similar observation was also made previously with a lesser purified preparation from calf thymus (Paik and Kim, 1968).

Isoelectric Point. The isoelectric point (p*I*) of protein methylase I was determined and a p*I* value of 5.1 was obtained by using a pH range of 3-10 Ampholine (figure not shown). Even though a single band was obtained, only 40-50% of the charged enzyme activity was recoverable after 30 h of electrofocusing due to its instability.

Specificity of the Enzyme. Table II lists the relative efficiency of various proteins as substrate for protein methylase I. Histones appear to be most active in accepting the methyl group from *S*-adenosyl-L-methionine. AI protein has been reported to be a good substrate for protein methylase I (Sundarraj and Pfeiffer, 1973); however, this protein in the

TABLE II: Relative Efficiency of Various Proteins as Substrate for Protein Methylase I.[a]

Protein Used as Methyl Acceptor	Source	% Efficiency
Histone		
Type II-A (crude)	Calf thymus	100.0[b]
H4(pure)	Calf thymus	68.0
H1 (pure)	Calf thymus	15.0
Protamine sulfate	Salmon	34.1
AI basic protein	Calf brain	30.0
Polyarginine		24.8
γ-Globulin	Human serum	18.7
Ribonuclease, oxidized	Bovine pancreas	13.3
Ribonuclease, "native"	Bovine pancreas	0

[a] Polylysine, lysozyme (egg white), trypsin (bovine pancreas), and albumin (bovine serum) had no substrate activity. [b] One hundred percent enzyme activity represents 35.0 pmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine used per min per mg of enzyme protein. Three milligrams of each protein was used. A more detailed procedure for enzyme assay is described under Materials and Methods.

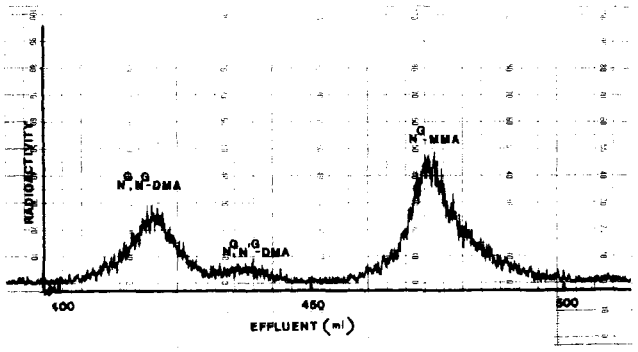

FIGURE 4: Radiogram of the end products of protein methylase I. One-half milliliter of 0.5 M phosphate buffer (pH 7.2), 0.5 ml of histone type II-A (60 mg), 0.5 ml of *S*-adenosyl-L-[*methyl*-^{14}C]methionine (50 nmol; 92 cpm/pmol), and 1.0 ml of purified protein methylase I (0.34 mg of protein) were incubated at 37 °C for 3 h. The methylated histone was treated as described under Materials and Methods and hydrolyzed at 110 °C for 48 h in 6 N HCl. HCl was removed, and the hydrolyzate was dissolved in 1.0 ml of water. One-tenth milliliter of this hydrolyzate was analyzed on an automatic amino acid analyzer equipped with a flow cell monitor of 60% counting efficiency. The flow rate was 45 ml per h. The column was first eluted with 0.38 N sodium citrate buffer, pH 5.84, at 24 °C, and after 2 h or at the tyrosine and phenylalanine position (approximately 90 ml of effluent), the buffer was changed to 0.35 N sodium citrate, pH 4.7. Since the height of radioactivity peaks in the figure does not give a quantitative amount of radioactivity, the cpm listed in Table I was obtained by using an integrator. More detailed experimental procedures are described under Materials and Methods.

present studies was found to be a poor substrate for the enzyme. This is probably due to either the fact that the Res-107 arginine in our AI protein preparation has already been partially methylated, or that enzyme preparation employed by others (Sundarraj and Pfeiffer, 1973) was enriched with AI-specific protein methylase (more detailed discussion will be presented later in this paper).

End Products of Protein Methylase I. When histone was methylated by purified protein methylase I with *S*-adenosyl-L-[*methyl*-^{14}C]methionine, and the acid hydrolyzate was analyzed by an automatic amino acid analyzer with flow cell monitor, the radiogram pattern shown in Figure 4 was obtained. N^G,N^G-Dimethylarginine (asymmetric), N^G,N'^G-dimethylarginine (symmetric), and N^G-monomethylarginine were the only radioactive methylated compounds. None of the methylated lysine derivatives was detected. This indicates that the purified enzyme preparation is completely free of protein methylase III activity. As mentioned earlier (Figure 1), protein methylase II activity was removed during Bio-Gel A-15m column chromatography.

Identity of Enzyme(s) to Form Various Methylated Arginines. The distribution of various methylated arginines in muscle protein is highly specific in regard to the kind of protein as well as the developmental stage of the animal. Reporter and Corbin (1971) observed the presence of N^G,N^G-dimethylarginine in the hydrolyzate of myosin isolated from developing leg muscle of rat and chicken. However, they were unable to find N^G-mono- or N^G,N'^G-dimethylarginine in the myosin and, when the animals developed into adults, N^G,N^G-dimethylarginine disappeared. This observation suggests that more than one enzyme is involved in the formation of methylated arginines. In order to resolve this question, we compared the ratio of various methylated arginines formed by protein methylase I preparations during the 120-fold purification of the enzyme; histone was methylated with *S*-adenosyl-L-[*methyl*-^{14}C]methionine by the enzyme preparation during each step of purification, the protein was hydrolyzed, and the radiogram was developed with the amino acid analyzer. As listed in columns 5–7, Table I, the ratio of radioactivity incorporated into the three methylated arginines was found to be quite constant. The result is in contrast to that of Reporter and Corbin (1971) since it strongly suggests that a single enzyme is responsible for the formation of all three methylated arginines. The highest amount of radioactivity is incorporated into N^G-monomethylarginine, followed by N^G,N^G-dimethylarginine, whereas the least amount of radioactivity was found in N^G,N'^G-dimethylarginine.

Identity of Enzyme(s) to Methylate Histone and AI Protein. The identity of the enzyme which methylates histone and AI protein is still somewhat controversial. Sundarraj and Pfeiffer (1973) and Jones and Carnegie (1974) presented evidence which indicates that a single enzyme is responsible for the methylation of histone and AI protein in vivo. On the other hand, Miyake (1975) reported that two enzymes are involved in the methylation of histone and AI protein. In order to investigate this problem further, we have compared the enzyme activity with both histone and AI protein as substrates during each of the various steps of enzyme purification. As shown in the last column of Table I, the ratio of substrate efficiency of histone and AI protein changed significantly during enzyme purification, suggesting that the methylation of these two protein substrates is carried out by two different enzymes. However, caution is necessary in the above conclusion for two reasons: (a) The ratio of substrate efficiency of histone vs. AI protein for protein methylase I depends on the amount of enzyme used (varying two- to five-fold as shown in Figure 3), and (b) the enzymatic product was not always tested for possible contamination with protein methylase III (lysine-residue methylating enzyme). Thus, it cannot strictly be ruled out that the changing ratio of histone vs. AI protein to a varying degree may reflect a difference in amount of enzyme or contamination with protein methylase III.

Initial Velocity Studies. The reaction mechanism of the protein methylase I from calf brain was investigated with studies of initial velocity and product inhibition. Since the entire amino acid sequence of histone H4 has been well characterized (Ogawa et al., 1969), it was chosen as the methyl accepting substrate protein. The arginine residues of histone H4 apparently are not methylated in vivo (Ogawa et al., 1969; DeLange et al., 1969).

When *S*-adenosyl-L-methionine was the varied substrate at fixed concentrations of histone H4, the initial velocity pattern shown in Figure 5 was obtained. The lines of the graph converge at one point on the abscissa. Similarly, when histone H4 was the varied substrate with different concentrations of *S*-adenosyl-L-methionine as the fixed substrate, a similar converging intersecting initial pattern was also obtained (figure not shown). It follows then that the reaction mechanism of protein methylase I is a sequential bireactant enzymic mechanism; this would exclude mechanisms such as the ping-pong. In this sequential mechanism, both substrates must be present simultaneously on the enzyme surface before any product is released. Cleland (1970) has shown that the following general form of the rate equation for such a sequential bireactant mechanism

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \qquad (2)$$

where v is the initial velocity, A (*S*-adenosyl-L-methionine) and B (histone H4) are substrate concentrations, V, K_a, K_b, and K_{ia} represent maximum velocity, Michaelis constants for

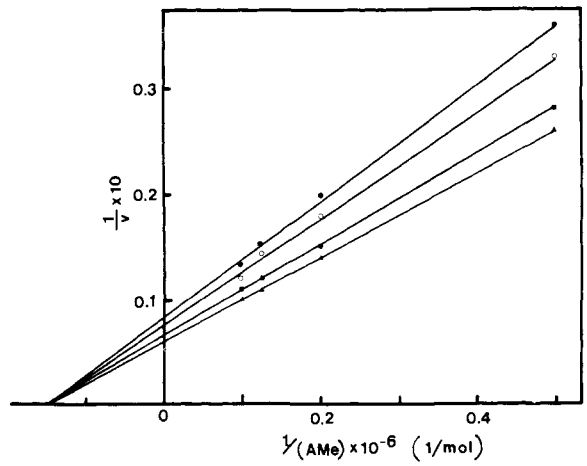

FIGURE 5: Effect of *S*-adenosyl-L-methionine on the protein methylase I reaction. *S*-Adenosyl-L-methionine concentration was varied from 2 to 10×10^{-6} M. The concentrations of histone H4 used: 0.53 (●), 0.8 (○), 1.6 (■), and 3.7 (▲) $\times 10^{-4}$ M. The purified enzyme preparation (0.034 mg) was used in the assay procedure described under Materials and Methods.

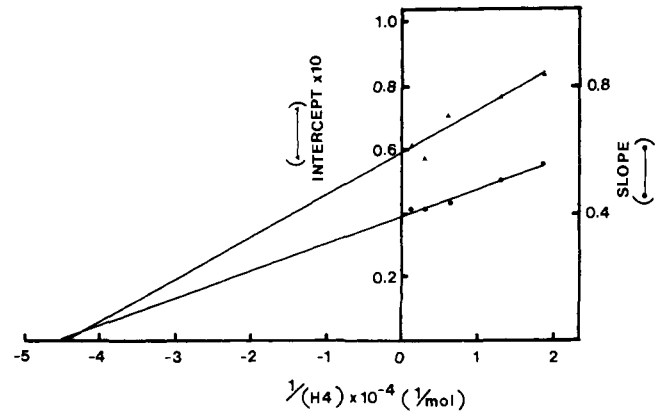

FIGURE 6: Secondary plots of the intercepts and slopes of Figure 5 against the reciprocals of the concentration of histone H4.

substrate A and B, and dissociation constant for the reaction of free enzyme with substrate A, respectively.

Kinetic Constants of Protein Methylase I from Calf Brain. The numerical values of the constants are extracted graphically by replotting the slopes and intercepts of the reciprocal plots (Figure 5) vs. the reciprocal concentrations of the nonvaried substrates. The secondary plot of the slopes and vertical intercepts obtained in Figure 5 is shown in Figure 6. Linear plots were derived. Although not shown, similar linearity was also obtained in secondary plots of different concentrations of histone H4 at varied fixed concentrations of *S*-adenosyl-L-methionine. From these secondary plots, various constants for protein methylase I were calculated and are given in Table III. The maximum velocity of the reaction was found to be 72.4 pmol of *S*-adenosyl-L-methionine consumed min^{-1} (mg of enzyme protein)$^{-1}$ at pH 7.2 and 37 °C.

Initial Velocity Studies in the Presence of Products. The initial velocity studies with two substrates such as shown in Figure 5 indicate that the protein methylase I reaction is a sequential mechanism; however, these data alone do not define whether the bireactants are ordered or random. However, Cleland (1970) pointed out that measurement of initial velocity in the presence of one of the reaction products can distinguish between the ordered Bi Bi mechanism and the random mechanism.

The type of inhibition expected depends on which substrate is varied and which product is the inhibitor, and full product inhibition studies involve the use of each product in turn as an inhibitor vs. each substrate as the variable one. However, because of experimental difficulties in isolating and purifying one of the reaction products, methylated histone H4, the reverse reaction, as well as product inhibition study with this protein, is presently impossible to carry out. Therefore, the product inhibition study was carried out with only *S*-adenosyl-L-homocysteine as the product inhibitor when *S*-adenosyl-L-methionine or unmethylated histone H4 was the variable substrate.

The product inhibition by *S*-adenosyl-L-homocysteine with *S*-adenosyl-L-methionine as the variable substrate appears to be competitive when the enzyme is saturated with histone H4 (figure not shown). This suggests that both reactants combine with the same enzyme form. A similar competitive inhibition was also observed when the enzyme was not saturated with histone H4 (figure not shown). On the other hand, when histone H4 is the variable substrate, the product inhibition pattern by *S*-adenosyl-L-homocysteine seems to be noncompetitive under conditions where the enzyme is not saturated with the other substrate, *S*-adenosyl-L-methionine (figure not shown).

TABLE III: Kinetic Constants for Protein Methylase I.

Constants[a]	Values (M)
K_a	7.6×10^{-6}
K_{ia}	5.9×10^{-6}
K_b	2.5×10^{-5}
K_{ib}	7.8×10^{-5}
K_i for *S*-adenosyl-L-homocysteine	2.62×10^{-6}

[a] K_a and K_b represent Michaelis constants for *S*-adenosyl-L-methionine and histone H4, respectively. K_{ia} and K_{ib} are the dissociation constants for the reaction of the free enzyme with substrate A (*S*-adenosyl-L-methionine) and the enzyme–*S*-adenosyl-L-methionine complex with substrate B (histone H4), respectively. K_i is the inhibition constant.

The inhibition patterns were analyzed further by replotting the slopes and intercepts vs. inhibitor concentration (figure not shown), and the K_i value for *S*-adenosyl-L-homocysteine was found to be 2.62×10^{-6} M (Table III).

Discussion

In the present paper, we have purified protein methylase I from calf brain approximately 120-fold with a 14% yield. The purified enzyme is free of any contamination by other protein methylases and endogenous substrate protein. Optimum pH is around 7.2 and the K_m value for *S*-adenosyl-L-methionine is 7.6×10^{-6} M which is close to that of partially purified calf thymus enzyme (Paik and Kim, 1968) (2.1×10^{-6} M). Since the intracellular concentration of *S*-adenosyl-L-methionine in various rat organs ranges between 10^{-4} and 10^{-5} M (Baldessarini and Kopin, 1966), the concentration of this methyl donor does not seem to be a limiting factor for enzymatic methylation in vivo. The K_m value for other protein-specific methyltransferases is around 10^{-6} M (Paik and Kim, 1975a,b). The K_i value of 2.62×10^{-6} M for *S*-adenosyl-L-homocysteine (which is a product inhibitor) is well above the intracellular concentration of 10^{-7} M (Salvatore et al., 1968). The fact that the K_m value for *S*-adenosyl-L-methionine and K_i for *S*-adenosyl-L-homocysteine are not too different suggests that these

TABLE IV: Comparison of Theoretical and Experimental Patterns of Product Inhibition.

Inhibitor	SAM as Varied Substrate[a]		H4 as Varied Substrate	
	H4 Unsaturated	H4 Saturated	SAM Unsaturated	SAM Saturated
	Theoretical Pattern			
Product P	NC	UC	NC	NC
Product Q	C	C	NC	
	Experimental Pattern			
Methylated H4	ND	ND	ND	ND
S-Adenosyl-L-homocysteine	C	C	NC	

[a] Abbreviations used: SAM, *S*-adenosyl-L-methionine; NC, noncompetitive; C, competitive; UC, uncompetitive; ND, not determined.

two components of the methylation reaction have a similar affinity for the enzyme.

An earlier experiment by Baldwin and Carnegie (1971) with crude protein methylase I isolated from rabbit brain indicated that the enzyme is highly specific for the Res-107 of arginine of AI protein isolated from human brain. However, the question as to whether a single enzyme is involved in the formation of the three methylated arginine derivatives (N^G,N^G-di-, N^G,N'^G-di-, and N^G-monomethylarginine) has remained unanswered until now. Deibler and Martenson (1973) could not detect the presence of N^G,N^G-dimethylarginine in any of the AI proteins isolated from various animal sources, while the other two methylated arginines were invariably present. Furthermore, Kakimoto and Akazawa (1970) reported that, although two dimethylated arginines were excreted, no N^G-monomethylarginine was detected in human urine. These observations, together with that of Reporter and Corbin (1971) that the methylation pattern of myosin is dependent on the developmental stage of the muscle, suggest that more than one enzyme might be responsible for the formation of these arginine derivatives. However, as shown in Table I, the ratio of radioactivity incorporation into the three methylated arginines by enzyme preparation during purification remains unchanged, suggesting that one enzyme is involved in the synthesis of N^G,N^G-di-, N^G,N'^G-di-, and N^G-monomethylarginine residues. Since the apparent molecular weight of the purified enzyme appears to be more than 1.5×10^6, it is highly tempting to speculate that the enzyme is composed of subunits and that the degree of polymerization might determine some of the anomalous observations cited above.

The biochemical significance of protein-arginine methylation remains an enigma. Since the methylated arginine residue (Res-107) of AI protein is in close proximity to the principal encephalitogenic determinant (Res-111 to Res-121) and methyl substitution of the guanidino group of arginine increases hydrophobicity (Tanford, 1962), it has been suggested that the enzymatic methylation of arginine Res-107 might play an important role in maintaining proper conformation of the myelin protein, thus facilitating conjugation between the myelin basic protein and hydrophobic lipid component (Baldwin and Carnegie, 1971). Since it is known that myelination of rat brain starts at about 10 days after birth (McIlwain, 1966), we have studied protein methylase I activity during early development of rat brain, using histone as substrate protein (Paik et al., 1972; Paik and Kim, 1973). The enzyme activity was found to be high in the fetal brain and decreased rapidly thereafter reaching approximately 50% of the fetal level before 10 days of life. Furthermore, myelin-deficient jimpy mice had normal amounts of protein methylase I activity in the brain. These results suggested that enzymatic methylation of myelin protein does not seem to play any direct role in the maturation of myelin.

On the other hand, Miyake (1975) studied the pattern of protein methylase I activity during early development of rat brain, using AI protein as substrate, and found that the increase of protein methylase I activity coincided with that of myelin formation. Since the results presented in Table I suggest that the enzyme which methylates AI protein might be different from that for histone, the data from our study (Paik et al., 1972b; Paik and Kim, 1973) should be reexamined, using the proper substrate protein, AI basic protein.

Table IV summarizes the results of the studies on product inhibition and these are compared with the theoretical pattern of product inhibition for an ordered Bi Bi enzymatic mechanism. Although the kinetic data presented in this paper are not complete, the results in Table IV suggest that the protein methylase I reaction is an ordered Bi Bi mechanism; *S*-adenosyl-L-methionine as the first substrate, histone H4 as the second substrate, methylated histone H4 as the first product released, and finally *S*-adenosyl-L-homocysteine as the second product released.

The reaction mechanism for protein methylase I is somewhat different from that of protein methylase II. The reaction mechanism for protein methylase II has been postulated to be random Bi Bi equilibrium whose rate-limiting step may be the interconversion of the central ternary complex (Jamaluddin et al., 1975).

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Purification and Properties of *Renilla reniformis* Luciferase†

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ABSTRACT: Luciferase from the anthozoan coelenterate *Renilla reniformis* (*Renilla* luciferin:oxygen 2-oxidoreductase (decarboxylating), EC 1.13.12.5.) catalyzes the bioluminescent oxidation of *Renilla* luciferin producing light (λ_B 480 nm, Q_B 5.5%), oxyluciferin, and CO_2 (Hori, K., Wampler, J. E., Matthews, J. C., and Cormier, M. J. (1973), *Biochemistry 12,* 4463). Using a combination of ion-exchange, molecular-sieve, sulfhydryl-exchange, and affinity chromatography, luciferase has been purified, approximately 12 000-fold with 24% recovery, to homogeneity as judged by analysis with disc and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel filtration, and ultracentrifugation. *Renilla* luciferase is active as a nearly spherical single polypeptide chain monomer of 3.5×10^4 daltons having a specific activity of 1.8×10^{15} $h\nu$ s^{-1} mg^{-1} and a turnover number of 111 μmol min^{-1} μmol^{-1} of enzyme. This enzyme has a high content of aromatic and hydrophobic amino acids such that it has an $\epsilon_{280nm}^{0.1\%}$ of 2.1 and an average hydrophobicity of 1200 cal $residue^{-1}$. The high average hydrophobicity of luciferase, which places it among the more hydrophobic proteins reported, is believed to account, at least in part, for its tendency to self-associate forming inactive dimers and higher molecular weight species.

The chemistry of the bioluminescent oxidation of a fully active synthetic analogue of *Renilla* luciferin by *Renilla* luciferase (*Renilla* luciferin:oxygen 2-oxidoreductase(decarboxylating), EC 1.13.12.5.) and the identity of the product excited state of this reaction are now known (Hori et al., 1973). The reaction path is illustrated in Figure 1.

Enzymological and structural studies on *Renilla* luciferase were not possible in the past due to isolation procedures which led to mixtures of active and inactive luciferase. Earlier preparations also apparently contained high levels of a single contaminating protein which was incorrectly characterized as luciferase (Karkhanis and Cormier, 1971). We have now overcome these difficulties partly by necessary changes in the initial handling and processing of the animals and partly by improved isolation procedures which include an affinity chromatography step. We report the isolation of *Renilla* luciferase, purified 12 000-fold to homogeneity, and the reexamination of the properties of the enzyme. In addition, we provide evidence that *Renilla* luciferase exists in its active form as a single polypeptide chain of 35 000 daltons and that this enzyme can self-associate to inactive higher molecular weight species.

Materials and Methods

Materials. Methanol and dimethylformamide were spectrophotometric or equivalent grade and, unless otherwise specified, all other chemicals used were reagent grade or the best quality available. All buffers and solutions were made up using deionized water or water twice distilled from glass having a maximum conductivity of 1.2 $\mu\Omega^{-1}$. DEAE[1]-cellulose was

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[1] Abbreviations used are: 2-ME, 2-mercaptoethanol; BSA, bovine serum albumin; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); DEAE, diethylaminoethyl; BSA, bovine serum albumin; DMF, dimethylformamide; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; TEMED, *N,N,N′,N′*,-tetramethylethylenediamine.