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Partial Purification and Characterization of a Protein Lysine Methyltransferase from Plasmodia of *Physarum polycephalum*†

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ABSTRACT: Plasmodia of *Physarum polycephalum* have an active protein lysine methyltransferase (*S*-adenosyl-L-methionine:protein-lysine methyltransferase, EC 2.1.1.43). This enzyme has been purified 40-fold with a 13% yield, and it catalyzes the transfer of methyl groups from *S*-adenosyl-L-methionine to the ε-amino group of lysine residues with formation of N^{ϵ}-mono-, N^{ϵ}-di-, and N^{ϵ}-trimethyllysines in a molar ratio of 4:1:1 based on [^{14}C]methyl incorporation into the methylated lysines. The ratio remains unchanged at all stages of the partial purification, as well as after fractionation by sucrose density gradient centrifugation and gel electrophoresis. The rate of protein methylation is time dependent, enzyme concentration dependent, and requires the presence of a sulfhydryl reducing agent for optimal activity. The enzyme has optimal activity at pH 8 and is inhibited by *S*-adenosyl-L-homocysteine and EDTA. Lysine-rich and arginine-rich histones serve as the most effective exogenous protein acceptors; *P. polycephalum* actomyosin is inactive, and chick skeletal myofibrillar proteins are 25% as effective as exogeneous mixed histones as substrates. Lysine, polylysine, ribonuclease A, cytochrome *c*, and bovine serum albumin are not methylated.

Protein methylation is observed in a wide variety of eucaryotic tissues, and many classes of proteins can be methylated, including actomyosins, histones, some ribosomal proteins, fungal and wheat germ cytochrome *c*, flagellin, and opsin (Paik & Kim, 1971, 1975). Protein methyltransferases specific for the N-methylation of lysine residues using *S*-adenosyl-L-methionine as the methyl donor have a wide distribution with the enzyme often associated with nuclei (Kim & Paik, 1965) and utilizing histones (Paik & Kim, 1970) as the preferred protein methyl acceptor. Nochumson et al. (1977) and Durban et al. (1978) have described the purification and characterization of an apparently soluble protein lysine methyltransferase from *Neurospora crassa* that preferentially uses unmethylated horse heart cytochrome *c* as a methyl acceptor.

We have shown previously that myosin prepared from plasmodia of *Physarum polycephalum* contains Lys(Me),[1] Lys(Me_2), Lys(Me_3), and Arg(G-Me_2) in a molar ratio of 1:1:2:2. *P. polycephalum* actin contains predominantly His-(3-Me) and small amounts of Lys(Me) and Lys(Me_2) (Venkatesan et al., 1975). The occurrence of these methylated basic amino acid residues in the contractile proteins of *P. polycephalum* encouraged us to examine further the methylation reactions in *P. polycephalum*. This paper describes the partial purification and characterization of a soluble enzyme which synthesizes protein-bound Lys(Me), Lys(Me_2), and Lys(Me_3) in a ratio of 4:1:1. A preliminary report of some of this work

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[1] Abbreviations used: Arg(G-Me), N^{G}-monomethylarginine; Arg(G-Me_2), N^{G}-dimethylarginine and $N^{G},N^{G'}$-dimethylarginine; DATD, diallyltartardiamide; His(3-Me), N^{3}-methylhistidine; MBAA, methylated basic amino acids; Lys(Me), N^{ϵ}-monomethyllysine; Lys(Me_2), N^{ϵ}-dimethyllysine; Lys(Me_3), N^{ϵ}-trimethyllysine; SAM, *S*-adenosyl-L-methionine.

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has appeared (Venkatesan & McManus, 1977).

Experimental Section

Materials

A-grade N^3-methylhistidine, N^ϵ-monomethyllysine hydrochloride, N^ϵ-dimethyllysine, N^ϵ-trimethyllysine, N^G-monomethylarginine, and $N^G,N^{G'}$-dimethylarginine were obtained either as free amino acids or as the sulfonate salts from Calbiochem. Free methylated amino acids were generated from the sulfonate salts by placing the derivatives on a 0.9 × 10 cm column of Dowex 1–acetate. The column was washed with water, and the free amino acid was recovered as the acetate salt in the eluate while the sulfonate was retained on the column.

Adenosine 5′-triphosphate disodium salt and histone type II-A were purchased from Sigma Chemical Co., and arginine-rich histones were obtained from Worthington Biochemical Corp. Ultrapure ammonium sulfate and density gradient grade sucrose were purchased from Schwarz/Mann; hematin, *S*-adenosyl-L-methionine iodide, B grade, and *S*-adenosyl-L-homocysteine, B grade, were from Calbiochem; acrylamide and bis(acrylamide) were from Eastman Kodak Co.; DEAE-cellulose and Sephadex gel were from Pharmacia Fine Chemicals, Inc.

L-[*methyl*-^{3}H]Methionine, 1.0 Ci/mmol, was purchased from International Chemicals and Nuclear Corp., Irvine, CA, and *S*-adenosyl-L-[*methyl*-^{14}C]methionine, 46 mCi/mmol, was obtained from Research Products International Corp.

Methods

Cultivation of P. polycephalum Microplasmodia. A culture of *P. polycephalum*, strain M_3c, spherules was supplied by Dr. Harold Rusch, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI.

A microplasmodial shake culture was started as follows. A small strip of filter paper covered with spherules was positioned on the walls of a 500-mL Erlenmeyer flask containing 25 mL of semidefined nutrient medium (Daniel & Rusch, 1961; Chin & Bernstein, 1968). The lower part of the filter paper just touched the meniscus of the medium. The flask with the spherules was placed in the dark at room temperature for 48 h. By this time plasmodial growth, as indicated by a yellow, moist appearance of the culture material, was apparent, and the flask was then put on an Eberbach shaker and shaken for 2–4 days in the dark until one could see microplasmodial growth in suspension. The microplasmodia appear as bright yellow round or oval beads, which settle out of the medium when the flask is not shaking. Two-liter Eberbach flasks containing 500 mL of the semidefined medium were incubated with 10 mL of a subcultured 3-day-old plasmodial suspension, and the flasks were placed in a New Brunswick incubator maintained at 22–26 °C and shaken at a rate of 110 reciprocations/min. The microplasmodia were generally harvested after 68–72-h growth by centrifuging for 5 min at 500 rpm (International PR-2 centrifuge). The microplasmodia were washed once with cold distilled water and weighed. Packed microplasmodia (100–200 g) were obtained from five Eberbach flasks.

Preparation of Subcellular Fractions from P. polycephalum. Microplasmodia were labeled by adding 100 μCi of L-[*methyl*-^{3}H]methionine (1 mCi/μmol) to 500 mL of medium at zero time, growing the plasmodia at 24 °C in shake culture, and harvesting after 72 h. The cells were washed with cold glass-distilled water and then homogenized in a Waring blender for 30 s in 20 volumes of 0.25 M sucrose, containing 0.2% Triton X-100 and 1 mM $CaCl_2$. After the foam settled, the homogenate was first spun at 50*g* for 10 min to remove unbroken cells and cell debris. It was then centrifuged for 10 min at 500*g* to obtain nuclei, the supernatant from this spin was centrifuged at 12000*g* for 30 min to recover a mitochondrial fraction, and, finally, the resulting supernatant fraction was centrifuged at 110000*g* for 60 min to separate the microsomal pellet from the soluble supernatant. The nuclear, mitochondrial, and microsomal pellets were washed 3 times with 0.25 M sucrose, suspended in 0.25 M sucrose, and then dialyzed, along with the soluble fraction, against 0.01 M Tris-HCl, pH 7.4, to remove sucrose and Triton X-100. Aliquots were taken for protein assay, radioactive analysis, and acid hydrolysis.

Isolation of Nuclei from Physarum. Nuclei were isolated using the method of Mohberg & Rusch (1971). Twenty grams of fresh microplasmodia was suspended in 200 mL of a solution containing 0.25 M sucrose, 0.01 M $CaCl_2$, 0.01 M Tris, pH 7.2, and 0.1% Triton X-100 and homogenized in a Waring blender for 30 s. After the foam settled, the homogenate was centrifuged at 50*g* for 5 min to remove cell debris, and then the supernatant was centrifuged for 10 min at 1000*g* in 50-mL conical polycarbonate tubes underlaid with 10 mL of 1 M sucrose containing 0.01 M $CaCl_2$, 0.01 M Tris, pH 7.5, and 0.1% Triton X-100. The pellet containing nuclei was resuspended in 70 mL of the isolation medium, underlaid with 1 M sucrose solution, and centrifuged again as before. Purity of the nuclear preparation was judged by observing several fields through a phase-contrast microscope.

Amino Acid Analysis of Protein. Protein samples (2–10 mg) were hydrolyzed in sealed glass ampules under N_2 gas in 6 N HCl at 110 °C for 48 h. Excess HCl was removed by repeated evaporation in vacuo, after addition of distilled water. The amino acids were taken up in 2 mL of glass-distilled water and mixed with 0.1 mL of a standard solution of amino acids containing 0.02 μmol of α-methyllysine, Lys(Me), Lys(Me_2), Lys(Me_3), His(3-Me), Arg(G,G′-Me_2), Arg(G-Me_2), and Arg(G′-Me). These were resolved by column chromatography as described by Morse et al. (1975), modified from the method of Deibler & Martenson (1973), using a Beckman amino acid analyzer, Model 119, fitted with a UR-30 ion-exchange column, 0.9 × 45 cm, and equipped with a stream divider to permit four-fifths of the effluent collected in a 2-min period to be diverted to vials for radioactive assay. Development was started with 0.545 N sodium citrate, pH 5.67, at 28 °C at a buffer flow rate of 46 mL/h. At 250 min, the buffer concentration was raised to 1.345 N Na^+ by the addition of NaCl, the pH was adjusted to pH 7.5, and the temperature was elevated to 55 °C. The run was stopped at 450 min.

Radioactive Assay Procedure. Radioactive samples were counted in a Packard Model 3375 liquid scintillation spectrometer with windows preset for optimal ^{14}C and ^{3}H countings. Aqueous samples, 0.1–1.5 mL, were counted in 10 mL of Triton X-100 counting mixture (one part of Triton X-100 to two parts of toluene, 5.5 g of PPO and 0.1 g of POPOP added per L). Counting efficiencies were 80 and 35% for ^{14}C and ^{3}H, respectively.

$NaDodSO_4$ Gel Electrophoresis in Acrylamide–DATD Gels. Proteins were incubated for 2 min at 100 °C in 0.2–0.5% $NaDodSO_4$–0.1% 2-mercaptoethanol (Fairbanks et al., 1971). Glycerol was then added to the samples (20–100 μg of protein) to a final concentration of approximately 10%, and the mixtures were applied to 0.6 × 10 cm 10% polyacrylamide–DATD (0.4%)–0.06% $NaDodSO_4$ disc gel columns and electrophoresed in a Buchler electrophoresis chamber. The gels were preelectrophoresed for 15 min at 1.5 mA/gel before application

Table I: Distribution of Methylated Basic Amino Acids in Subcellular Fractions of *P. polycephalum*[a]

fraction	total protein (mg)	total cpm in protein $\times 10^{-7}$	cpm in MBAA per mg of protein	mole ratio					
				Lys-(Me)	Lys-(Me_2)	Lys-(Me_3)	His-(Me)	Arg-(G-Me_2)	Arg-(Me)
homogenate[b]	1800	3.98	470	1	0.8	1.75	0.5	1.5	0.3
nuclear fraction	125	0.3	1591	1	1.3	2.0	0.3	0.6	2.0
mitochondrial fraction	65	0.14	1021	1	2.5	5.5	0.8	0.8	0.3
microsomal fraction	219	0.7	1071	1	0.8	1.6	0.5	2.0	0.2
soluble	720	1.48	604	1	0.7	2.0	0.6	0.5	1.0

[a] Microplasmodia were grown in 500 mL of medium containing 100 μCi of L-[*methyl*-^{3}H]methionine (1 mCi/μmol) at 24 °C for 72 h. [b] Homogenization medium: 0.25 M sucrose with 0.2% Triton X-100 and 1 mM $CaCl_2$. Radioactive microplasmodia were homogenized for 30 s in a Waring blender with 1:20 volumes of homogenizing medium. The nuclear, mitochondrial, and ribosomal pellets were washed with 0.25 M sucrose 3 times before use. All fractions were dialyzed to remove sucrose and Triton X-100 before protein and radioactive analysis. See Methods for further details.

of the samples. The chamber buffer consisted of 0.05% Na-$DodSO_4$ and 5% glycerol in 0.025 M Tris–glycine, pH 8.8. The samples were applied with bromophenol blue as the tracking dye, and the volume of the sample did not exceed 200 μL. Electrophoresis was performed at 1.2 mA/gel tube and was terminated when the tracking dye reached the bottom of the gel. The proteins were fixed and stained in 0.02% Coomassie blue in 50% methanol–7.5% acetic acid and destained by repeating washing in 10% methanol–7.5% acetic acid. The gels were scanned by using a Gilford gel scanner at an absorbance of 540 nm.

Non-NaDodSO_4 Polyacrylamide Gel Electrophoresis. Electrophoresis of protein samples (10–100 μg) was performed by using polyacrylamide disc gels, 0.6 × 9 cm, prepared with 7.5% acrylamide–0.2% bis(acrylamide) at a constant current 2 mA/tube with 0.025 M Tris–glycine buffer, pH 8.3, as a chamber buffer. The run was stopped when the tracking dye front had advanced to within 1 cm of the end of the gel. The proteins were fixed and stained in 0.02% Coomassie blue in 50% MeOH–7.5% acetic acid.

Assay of Protein Lysine Methyltransferase. Protein methyltransferase activity was assayed in fractions prepared from *P. polycephalum* plasmodia by measuring the incorporation of ^{14}C from *S*-adenosyl-L-[*methyl*-^{14}C]methionine into methylated basic amino acid residues of exogenous protein. The standard incubation mixture contained 0.1 mL of 0.1 M KCl, 0.05 mL of 0.1 M $MgCl_2$, 0.1 mL of 0.1 M β-mercaptoethanol, 0.1 mL of 1.0 M Tris, pH 8.0, 0.1 mL of histone, type II-A (3 mg), 0.25 μmol of SAM-I containing 0.5 μCi of [^{14}C]SAM, enzyme solution in 0.01 M Tris buffer, pH 7.8, and glass-distilled water to a final volume of 1.0 mL. The reaction mixture was incubated for 30 min at 37 °C in a water bath, and the reaction was stopped by addition of 1 mL of 40% cold Cl_3AcOH.

The proteins were collected by centrifuging the suspension for 20 min at 500*g*. The Cl_3AcOH-precipitated proteins were then subjected to several washings to remove nucleic acids, lipids, and phospholipids (Allfrey et al., 1964) including two 15-min washings in boiling 10% Cl_3AcOH, followed by one wash with cold 10% Cl_3AcOH, two washes at 50 °C with 95% ethanol, one wash at room temperature with an ethanol–chloroform–ether mixture, ratio 2:2:1, and, finally, one wash with ethyl ether. The dried protein was then dissolved in 0.5 N NaOH and assayed for protein by using the Lowry method (Lowry et al., 1951), and an aliquot was then taken for radioactive measurement. The remainder was hydrolyzed in 6 N HCl and used for assay of ^{14}C-methylated amino acids. Results are expressed in terms of counts per minute of ^{14}C incorporated into acceptor protein per milligram of enzyme protein per 30 minute or as picomoles of ^{14}C-methylated amino acid residues synthesized per milligram of enzyme protein.

Results

Subcellular Location of Methylated Proteins in P. polycephalum. Earlier studies (Venkatesan et al., 1975) aimed at examining the pattern of methylation of purified actin and myosin obtained from macro- and microplasmodia of *P. polycephalum* had documented the presence of small amounts of Lys(Me) and Lys(Me_2) in addition to one residue of His-(3-Me) in actin and Lys(Me), Lys(Me_2), Lys(Me_3), and Arg(G-Me_2) in myosin. As shown in Table I, methylated basic amino acids are also detected in other protein fractions, as indicated by the distribution of ^{3}H-methylated basic amino acids residues in subcellular fractions isolated from microplasmodia of *P. polycephalum* that were prelabeled with 100 μCi of L-[*methyl*-^{3}H]methionine for 72 h in 500 mL of medium. All of the above ^{3}H-labeled amino acids are demonstrated in these fractions with clear differences in the mole ratios of these residues in the different fractions. Noteworthy is the occurrence of relatively high levels of Lys(Me_3) in the mitochondrial fraction. Since no efforts were made to purify any of these fractions, extensive cross contamination among the fractions is probable, obviating quantitative interpretation of the distribution patterns. However, these preliminary results showed that methylation was a significant modification of *P. polycephalum* proteins and made it attractive to examine *Physarum* protein methyltransferase activities.

Protein Methyltransferase in P. polycephalum. Protein methyltransferase activity was assayed in crude extracts prepared from *P. polycephalum* microplasmodia by using [^{14}C]-SAM as the methyl donor. Histones (histone type II-A; Sigma) were used as the acceptor protein for routine in vitro assays since histones have been shown to serve as effective substrates for protein methylases from a variety of sources (Paik & Kim, 1975). The crude extract was prepared by centrifuging a homogenate, prepared as described below, consisting of 1:3 (w/v) microplasmodia in 0.05 M KCl, 0.01 M Tris, pH 7.8, and 1 mM DTT, for 10 min at 3000*g*. A total of 4320 cpm of ^{14}C was incorporated into acceptor protein per mg of extract protein per h by using standard assay conditions (see Methods), and the pattern of incorporation of radioactivity into basic amino acid residues in an acid hydrolysate of the washed proteins is illustrated in Figure 1. More than 50% of the total incorporated radioactivity is found in Lys(Me), Lys(Me_2), Lys(Me_3), Arg(N-Me_2), Arg(N-Me), and an unknown region which does not chromatograph with any of the standard basic amino acids. The low level of radioactive Arg(N-Me_2) and Arg(N-Me) does suggest the presence of a protein arginine methyltransferase, but this activity and the unknown radioactive peak disappear at an early stage during

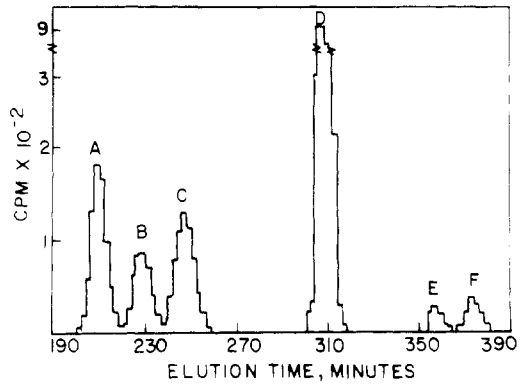

FIGURE 1: Profile of ^{14}C incorporation into methylated basic amino acids obtained from the acid hydrolysate of proteins after in vitro incubation in a standard assay with *Physarum* extract. The protein hydrolysate was analyzed on a Beckman amino acid analyzer as described previously (Morse et al., 1975). (A) Lys(Me); (B) Lys-(Me_2); (C) Lys(Me_3); (D) unknown; (E) Arg(Me_2); (F) Arg(Me).

purification of the protein lysine methyltransferase.

When the homogenate was centrifuged at 128000*g* for 45 min, the protein lysine methyltransferase activity was recovered in the supernatant with a threefold increase in specific activity. The distribution of radioactivity among the methylated lysines obtained from a protein hydrolysate following incubation with this soluble extract was 46% in Lys(Me), 24% in Lys(Me_2), and 31% in Lys(Me_3). When the incorporation is expressed as a mole ratio, it is apparent that Lys(Me) is the major methylated species. Insignificant incorporation of radioactivity occurred in the absence of added histones.

The effect of time of incubation on distribution of ^{14}C incorporated into the three methylated lysines was examined by using the high-speed extract. The percent distribution of ^{14}C was determined by using assay mixtures incubated for 5, 15, 30, 60, and 120 min. The percent recovery of the total radioactivity incorporated into the three methyllysines isolated from an acid hydrolysate of the acceptor protein was between 45 and 49%, and the percent distribution of radioactivity in the methylated lysines was 40.7–44.3% in Lys(Me), 19.3–23.4% in Lys(Me_2), and 33.3–36.6% in Lys(Me_3) over the 5–120-min period.

The residue from the 3000*g* centrifugation showed a low level of protein lysine methyltransferase activity, and it was of interest to examine nuclei as a possible enzyme source. Purified nuclei were obtained by the method of Mohberg & Rusch (1971), suspended in 4 volumes of 0.25 M sucrose, 0.01 M Tris-HCl, pH 7.8, 0.01 M EDTA, and 1 mM DTT and briefly sonicated. The nuclear extract, containing 1.8 mg of protein per mL, was assayed for protein lysine methyltransferase activity by using conditions identical with those used for assaying the soluble enzyme activity. After a 30-min incubation at 37 °C, the incorporation and distribution of ^{14}C in the methyllysines of acceptor protein were determined. Low activity, 112 cpm, was detected in methylated lysines per milligram of nuclear protein extract, and the percent distribution of ^{14}C was 35% in Lys(Me), 35% in Lys(Me_2), and 28% in Lys(Me_3). No activity was detected in the Arg(G-Me_2) area.

Partial Purification of the Enzyme. Since preliminary studies of the properties of soluble *P. polycephalum* protein lysine methyltransferase indicated that the enzyme was relatively stable, it was feasible to develop a purification procedure. The process was aided by the observation that incorporation of ^{14}C from [^{14}C]SAM into methylated arginine residues and into the unknown region disappeared after the original enzyme extract had been fractionated with ammonium sulfate. The 35–60% ammonium sulfate fraction catalyzed only the methylation of lysine residues, and essentially all radioactivity measured in the washed protein recovered from an incubation mixture was accounted for in the methylated lysines. Thus, the purification scheme deals exclusively with a protein lysine methyltransferase activity. A typical purification procedure is presented here. All steps were carried out at 0–4 °C. Fresh or frozen *P. polycephalum* microplasmodia (100 g wet weight) were suspended in 1 volume of medium consisting of 0.2 M KCl, 0.02 M Tris-HCl, pH 7.6, and 0.1% β-mercaptoethanol (medium A) and homogenized in an ice-cooled omnimixer set at speed 6 for three 3-s bursts. The foamy bright yellow-orange homogenate was centrifuged for 60 min at 80000*g*, and the clear yellow-orange viscous supernatant was removed. All further steps were performed at 0–4 °C. The supernatant was adjusted to pH 7.5, and 0.1 volume of 2% protamine sulfate was added slowly with continuous stirring. After stirring for 30 min, we removed the flocculent precipitate by centrifugation of the suspension for 30 min at 17300*g*. The supernatant, about 150 mL, was removed from the packed precipitate, and a saturated solution of ammonium sulfate (saturated at room temperature and adjusted to pH 7.2) was added slowly with constant stirring to a final concentration of 35% (v/v). After stirring for 30 min, we centrifuged the suspension for 30 min at 17300*g*. The precipitate was saved for enzyme assay, and saturated ammonium sulfate was added to the supernatant to a final concentration of 60% as described above. After stirring for 30 min, we collected the precipitate by centrifuging at 17300*g*, and the supernatant was either reserved for assay or discarded. The precipitate from the 35–60% fraction was dissolved in about 20 mL of 0.1 M KCl, 0.01 M Tris-HCl, pH 7.6, and 0.1% β-mercaptoethanol (medium B) by gently mixing in a Dounce homogenizer. This solution was dialyzed for 16 h against medium B with two changes of this buffer in preparation for applying the material to a DEAE-cellulose column, 2.5 × 10 cm, that was equilibrated with medium B.

The dialyzed sample, usually 2–25 mL, was applied to the column, and the column was developed by stepwise application of two bed volumes each of medium B containing 0.1, 0.2, 0.3, 0.4, and 0.5 M KCl. The enzyme was eluted with 0.3 and 0.4 M KCl. The active fractions were pooled and dialyzed overnight against medium B in preparation for applying the enzyme-containing solution to a Sephadex G-200 column. About 60 mg of protein was loaded onto this column, 2.5 × 84 cm, which was equilibrated with medium B and developed with the same buffer. Fractions (8 mL) were collected at an upward flow rate of 23 mL/h. Active enzyme was collected by starting at 250 mL and eluting through to 350 mL. This region was pooled and the enzyme was concentrated with a Diaflo apparatus using a PR-10 filter.

The concentrated active enzyme could be stored in ice and retained essentially full activity under these conditions for at least 10 days. Table II summarizes the results of this partial purification which gives a 40-fold increase in the specific activity of the methyltransferase and a 13% yield. At no stage in this purification was there clear evidence for a systematic variation in the percent distribution of radioactivity incorporated into the three methylated lysines synthesized in reactions catalyzed by the various fractions, and, at all stages, Lys(Me) accounted for over 40% of the total radioactivity found in the methylated lysines.

Some Properties of the Protein Lysine Methyltransferase. The usual extraction medium for preparation of the soluble extract consisted of an equal volume of 0.2 M KCl, 0.02 M

Table II: Partial Purification of *P. polycephalum* Protein Lysine Methyltransferase[a]

enzyme fraction	vol (mL)	protein (mg/mL)	total protein (mg)	sp act. [pmol/(min mg of enzyme protein)]	sp act. [cpm in $Lys(Me_x)$ per mg of protein]	yield (%)	purifn (x-fold)	% distribution Lys-(Me)	Lys-(Me_2)	Lys-(Me_3)
homogenate	300	10.6	3180	6.3	948	100	1	42	26	23
soluble extract	180	8.2	1476	16.4	2459	120	2.6	42	29	29
35–60% (v/v) $(NH_4)_2SO_4$ ppt	50	10	500	28.6	4344	71	4.5	45	25	30
0.4 M KCl eluate from DEAE-cellulose column	40	2.3	92	81.3	12250	37	13	48	26	26
eluate from Sephadex G-200 column	10	1.0	10	257.3	38776	13	40	41	25	34

[a] Details of the fractionation procedure are described under Results.

Tris-HCl, pH 7.6, and 0.1% β-mercaptoethanol added to washed plasmodia. Increasing the concentration of KCl in the buffered medium up to 0.5 M KCl has no effect on total incorporation of ^{14}C into the basic amino acid residues or on the distribution of radioactivity in the methylated lysines. However, omission of a reducing agent during the initial extraction procedure and in enzyme assays inhibits ^{14}C incorporation by at least 50%, although the ^{14}C distribution is unchanged. Treatment of the extract with RNase A (100 μg/mL) or 0.02% protamine sulfate has no effect on the pattern of methylation.

The enzyme reaction using the standard assay conditions is linear with time up to at least 15 min and begins to plateau by 30 min. The reaction velocity is proportional to the amount of enzyme protein added up to at least 0.5 mg of protein by using a 35–60% ammonium sulfate protein fraction which represents a fivefold purification of the enzyme (Table II). Enzyme activity is heat labile, and the activity is reduced to less than 3% of maximal activity after heating for 3 min at 60 °C. This reduction in activity is not altered by addition of SAM or exogenous histone. However, added histone does protect against inactivation when the enzyme is heated to 45 °C for 5 min. In the absence of substrate, 50% of the activity is lost within 5 min, and a residual activity equivalent to about 28% of the maximal activity persists for up to 20 min. When histone is added during this treatment, no activity is lost after 5 min at 45 °C, and 35% of the maximal activity is still demonstrated after 20 min. Added SAM (2 μmol/mL of enzyme fraction) is without effect on the course of the inactivation.

Maximal activity is observed at pH 8 by using either Tris or phosphate buffers. At pH 7, the distribution of radioactivity in the methylated lysines is not altered, although the total incorporation of ^{14}C into total protein is depressed 50% and the percent of total radioactivity in the methyllysines is reduced to 27%.

Both the crude and purified enzymes have a high affinity for the substrate *S*-adenosyl-L-methionine. An apparent K_M of 7.3×10^{-6} M was calculated for *S*-adenosyl-L-methionine, a value which is characteristic of a variety of other protein methyltransferases (Paik & Kim, 1975). The activity of the enzyme is inhibited 70% by 0.12 μM *S*-adenosylhomocysteine. Although no clear dependence on the presence of divalent ions for activity of the enzyme has yet been shown, the addition of 10 mM EDTA effected a 64% inhibition of activity.

Substrate Specificity of the Protein Lysine Methyltransferase. The relative capacity of various proteins to serve as substrates for the partially purified enzyme is shown in Table III. The results show that the enzyme does exhibit specificity toward some substrates in vitro, especially the histones, with lysine-rich histones being more active in accepting the methyl group of SAM than arginine-rich histones in vitro. Myofibrillar protein prepared from embryonic chicken skeletal muscle showed about 25% of the acceptor activity compared to crude histone while *P. polycephalum* actomyosin showed no acceptor activity in this system. It should be mentioned that little or no endogenous substrate activity was demonstrated either with homogenate or by using the purified enzyme. Bovine serum albumin, free lysine, and polylysine did not serve as substrates for the enzyme, while ribonuclease A and horse cytochrome *c* exhibited minimal capacities as possible protein acceptors.

Table III: Efficiencies of Various Proteins as Substrates for Protein Lysine Methyltransferase[a]

protein used as methyl acceptor	% efficiency[b]
histone, type II-A, crude	100
slightly lysine-rich histone	1000
arginine-rich histone	330
histone, type II-A, 100 °C for 10 min	75
myofibrillar fraction, chick muscle	25
ribonuclease A	7
cytochrome *c*	3

[a] *P. polycephalum* actomyosin, bovine serum albumin, polylysine, and lysine had no substrate activity. Three milligrams of each protein was used. [b] One hundred percent enzyme activity represents 82 pmol of *S*-adenosyl-L-[*methyl*-^{14}C]methionine taken up per min per mg of enzyme protein. See Methods for a detailed description of the assay conditions.

Behavior of the Partially Purified Protein Lysine Methyltransferase on a Sucrose Density Gradient and Gel Electrophoresis. Partially purified enzyme (11 mg) obtained from a Sephadex G-200 column was layered onto a 5–20% sucrose gradient in 0.1 M KCl and 0.01 M Tris, pH 7.6, containing 0.1% β-mercaptoethanol and centrifuged in a Beckman Model L2-65B centrifuge with an SW27 rotor at 25000 rpm for 16 h. Approximately 2-mL fractions were collected by puncturing the bottom of the tube. Aliquots (0.2 mL) for protein and aliquots (0.5 mL) for enzyme assays were removed from each fraction. The profiles obtained following gradient fractionation and assay for protein and methyltransferase activity are illustrated in Figure 2. The methyltransferase activity is found near the top of the gradient. Fraction 21 represents the peak activity while most of the protein is found between fractions 15 and 20, resulting in a threefold increase in specific activity over the Sephadex G-200 fraction layered on the gradient. A total of 60 mg of the purified enzyme was treated in this fashion.

The sucrose gradient fractions containing the highest enzyme activity (fractions 20 and 21) were concentrated, and 90-μg samples were electrophoresed on 7.5% polyacrylamide disc gels. One of these gels was stained with Coomassie blue, destained, and scanned for protein. The remainder were di-

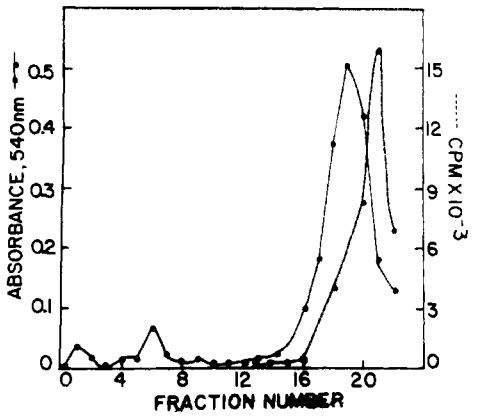

FIGURE 2: Profile of protein and methyltransferase activity obtained from sucrose density gradient centrifugation. A pooled enzyme fraction (11 mg) obtained from a Sephadex G-200 column was layered onto a 5–20% sucrose gradient in 0.1 M KCl, 0.01 M Tris, pH 7.6, and 0.1% β-mercaptoethanol and centrifuged in an SW27 rotor in a Beckman L2-65B centrifuge at 25 000 rpm for 16 h. Fractions (2 mL) were collected from the bottom of the centrifuge tube. 0.2-mL aliquots were used for protein assay and 0.5-mL aliquots were used for enzyme assay.

vided into five sections and extracted overnight with 5% sucrose containing 0.01 M KCl, 0.01 M Tris, pH 8.0, and 0.1% β-mercaptoethanol. The extracts were then assayed for enzyme activity. As is seen in Figure 3, essentially all of the enzyme activity coincided with the protein contained in gel section 4. When the enzyme activity recovered in gel section 4 was calculated in terms of specific activity, this material had a specific activity of 6769 pmol of SAM incorporated per mg of protein, representing about a 1000-fold increase in methyltransferase activity over the specific activity of the original homogenate. Acid hydrolysis of the protein recovered from assay of methyltransferase activity in gel fraction 4 showed the ^{14}C incorporation to be in Lys(Me), Lys(Me_2), and Lys(Me_3) in approximately the same ratio as that which was seen during earlier purification steps.

In an effort to get some estimate of the complexity of the protein fraction recovered from gel section 4, we electrophoresed this material on a 10% polyacrylamide–DATD–Na$DodSO_4$ gel, and, following staining of the gel with Coomassie blue, we scanned the gel at 540 nm. A minimum of three protein components was noted with approximately 45% of the total protein corresponding to a molecular size of approximately 32 000 as estimated from standard protein mobility curves. The remaining protein fractions, making up 29 and 26% of the total protein, had mobilities corresponding to molecular sizes of approximately 62 000 and 100 000, respectively. It remains to be determined whether or not any of these fractions correspond to or contain the protein lysine methyltransferase.

Discussion

Protein lysine methyltransferases have been demonstrated from a variety of sources, including calf thymus nuclei (Paik & Kim, 1970), 9-day-old embryonic chick nuclei (Greenaway & Levine, 1974), rat brain nuclei (Wallwork et al., 1977), rat kidney nuclei (Paik et al., 1973), *Escherichia coli* ribosomes (Chang et al., 1975), and cytosol of *N. crassa* (Nochumson et al., 1977). The predominantly nuclear origin of this enzyme was a matter of concern in considering the cellular localization of the *P. polycephalum* protein lysine methyltransferase described in this paper. The enzyme has been purified as a soluble enzyme, but the possibility existed that the *Physarum* enzyme may in fact have a nuclear origin. However, the

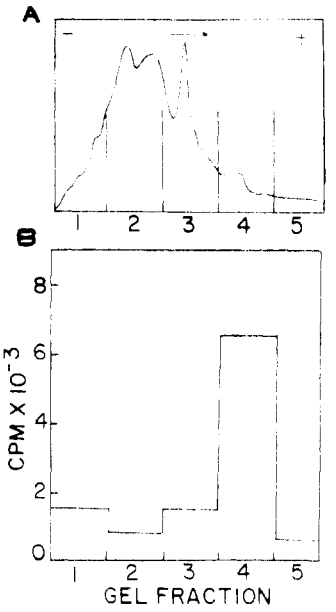

FIGURE 3: (A) Protein pattern obtained following electrophoresis of 90 μg of protein obtained from pooled fractions 20 and 21 from sucrose density gradient (see Figure 2). Electrophoresis was performed by using 7.5% acrylamide disc gels in 0.025 M Tris–glycine buffer, pH 8.3, at 4 °C at 2 mA/tube. A gel was stained with Coomassie blue and scanned by using a Gilford gel scanner at 540 nm. (B) Protein was recovered from unstained gels which were divided into five sections; protein was eluted and assayed for enzyme activity.

procedure used in the preparation of the initial homogenate, coupled with the observation that the nuclei of *P. polycephalum* are relatively difficult to disrupt (Mohberg & Rusch, 1971), renders the possibility of significant leakage or nuclear contribution to the cytosol unlikely. Moreover, when purified nuclei were sonicated and the nuclear extract was used as the enzyme source, the activity was approximately one-third of that obtained when the cytosol fraction was assayed. The distribution of radioactivity incorporated into the methylated lysine residues as a consequence of transfer of methyl groups from *S*-adenosyl-L-[*methyl*-^{14}C]methionine to the acceptor histone differed from that found when the soluble preparation was the enzyme source. Thus, although an activity capable of catalyzing the methylation of lysine residues can be demonstrated in *Physarum* nuclei, it is probably not identical with that of the soluble protein lysine methyltransferase.

The *Physarum* cytosol enzyme has been purified 40-fold by conventional procedures, and a 1000-fold purification was achieved when the 40-fold purified enzyme was subjected to sucrose density gradient centrifugation and gel electrophoresis. This represents a significantly higher degree of purification than has been achieved for the enzyme from any of the nuclear sources, but it is still well below that reported for the soluble cytochrome *c* specific protein lysine methyltransferase isolated from *N. crassa* (Durban et al., 1978). This enzyme has been purified approximately 3500-fold by steps including preparative gel electrophoresis and it is about 80% pure. Na$DodSO_4$ gel electrophoresis of the *Physarum* enzyme obtained after analytical gel electrophoresis shows the presence of multiple protein species, and further work is needed to achieve a higher purity and to characterize further the active species.

Although low levels of radioactive Arg(G-Me_2) and Arg(G-Me) were synthesized on exogenous histone in the presence of *Physarum*-soluble extract and *S*-adenosyl-L-[*methyl*-^{14}C]-methionine in addition to the three methylated lysines, the capacity to synthesize the methylated arginines disappeared at an early stage of purification, and an outstanding feature

of the formation of the methylated lysines is the general constancy of the amounts of newly synthesized methylated lysines. Within experimental limits, a ratio of 4:1:1 for Lys(Me), Lys(Me_2), and Lys(Me_3) persists throughout the purification and in assays performed by using incubation times of from 5 to 120 min. This ratio differs markedly from that seen in studies performed with the cytochrome *c* specific enzyme of *N. crassa* in which the products synthesized were Lys(Me), Lys(Me_2), and Lys(Me_3) in a ratio of 1:3:4 (Durban et al., 1978). This ratio was constant throughout purification, and the authors invoked this constancy as suggestive of a single enzyme being responsible for synthesis of all three lysines. This *N. crassa* purified enzyme does not catalyze transfer of methyl groups from SAM to added histone, and its optimum pH is 9, distinguishing it from the *Physarum* enzyme. However, the apparent K_M for SAM is the same order of magnitude for both enzymes, and both are inhibited by *S*-adenosyl-L-homocysteine. These latter properties are features common to most protein methyltransferases (Paik & Kim, 1975). The ribosomal protein lysine methyltransferase from *E. coli* studied by Chang et al. (1975) is another example of a nonnuclear lysine-specific enzyme, and it is of interest that, in contrast to the constant ratios of newly synthesized methylated lysines observed by using *Physarum* enzyme and the *N. crassa* enzyme, the ribosomal enzyme synthesized relatively greater amounts of Lys(Me) as the enzyme was purified. This change in the relative level of Lys(Me) as a function of enzyme purity could be used as evidence for the existence of more than one protein lysine methyltransferase, each responsible for the successive addition of methyl groups on the ϵ-amino group of a specific lysine residue. In general, the methylated lysine species contained in various proteins is highly specific; e.g., Lys(Me_3) is the major methylated lysine in rabbit skeletal muscle myosin (Kuehl & Adelstein, 1970; Morse et al., 1975) and in cytochrome *c* isolated from wheat germ and *N. crassa* (DeLange et al., 1969, 1970). Lys(Me) and Lys(Me_2) may occupy positions 9 and 27 of pea embryo histone H3 (Patthy et al., 1973) and are found in opsin of bovine retina (Reporter & Reed, 1972). The uneven distribution of methylated lysine residues in various proteins suggests the involvement of multiple enzymes, and additional information on specific acceptors and properties of purified protein lysine methyltransferases will be necessary in order to resolve this question.

The question of the nature of the natural substrate for the *P. polycephalum* protein lysine methyltransferase remains to be answered. Exogenous mixed histone type II-A from calf thymus was used routinely as the substrate protein throughout the enzyme purification and, as Table III shows, histone, particularly lysine-rich histone, is the most effective acceptor. The *Physarum* enzyme is devoid of endogenous substrate activity, although a wide spectrum of N-methylated proteins is distributed in subcellular fractions of *Physarum* microplasmodia. It is probable that the natural acceptor is already specifically methylated and retains its tertiary native structure in vitro, thereby preventing further methylation catalyzed by the methyltransferase. A heterologous substrate, such as the calf thymus histone preparation, may therefore serve as a more effective in vitro substrate by virtue of having additional acceptor sites available.

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