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IDENTIFICATION OF A SOLUBLE PROTEIN METHYLASE IN CHICKEN EMBRYO NUCLEI

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SUMMARY

A protein methylase, which methylates the ε-amino group of lysine residues, was found in soluble extracts of chicken embryo nuclei. The enzyme was partially purified by chromatography on CM-cellulose but the final preparations were still capable of endogenous methylation (methylation without added acceptor molecules). The methylase was optimally active at pH 8.4 and required Mg^{2+} and dithiothreitol for maximum activity. The rate of protein methylation was dependent on the time of incubation and on the enzyme concentration. Methylation was stimulated by calf-thymus histones and was inhibited by *S*-adenosyl-L-ethionine and *S*-adenosyl-L-homocysteine. The K_m value of the enzyme for *S*-adenosyl-L-methionine was $3.06 \cdot 10^{-6}$ M.

INTRODUCTION

Protein methylation has been observed in a wide variety of eukaryotic tissues [1] and three classes of protein methylases have been identified [2–4]. Protein methylases I and II, which methylate arginine residues and protein carboxyl groups, respectively, are present in the cytoplasm [2, 3] whereas protein methylase III, which methylates lysine residues, is found in the nucleus [4]. Acceptor proteins are methylated after synthesis and the methylases all use *S*-adenosyl-L-methionine as the methyl group donor.

Many classes of proteins are capable of being methylated. Although this suggests that protein methylation fulfills several functions, the full biochemical significance of this modification reaction is not yet understood. The suggestions that the methylation of chromosomal proteins affects cellular differentiation, gene activity and chromosome structure are of particular interest [1] and elevated protein methylase activities have been observed in a variety of proliferating cell systems [5–8]. In this paper we describe some of the properties of a protein methylase isolated from nuclear extracts of chicken embryo cells. The enzyme is soluble and is therefore amenable to biochemical analysis. Chicken embryo cells are undergoing rapid development and have a high metabolic rate; they are therefore potentially useful for studying the activity and possible functions of protein methylases during embryogenesis and subsequent development.

MATERIALS AND METHODS

Materials

9-day-old embryonated chicken eggs (cofal negative) were obtained from Spafas Incorporated, RFD No. 9, Norwich, Conn., U.S.A. *S*-Adenosyl-L-[*Me*-^{3}H]-methionine (specific radioactivity, 8.02 Ci/mmole] and Aquasol scintillation solution were purchased from New England Nuclear, Boston, Mass., U.S.A. *S*-Adenosyl-L-homocysteine, *S*-adenosyl-L-ethionine, *N*-ε-methyl-L-lysine, L-3-methylhistidine, dithiothreitol and calf-thymus histones (Type II-a) were all obtained from Sigma. Bovine serum albumin and pronase were from Calbiochem. Sephadex G-150 was purchased from Pharmacia Fine Chemicals Ltd, Piscataway, N.J., U.S.A.; CM-cellulose (CM 52 cation exchanger) and Whatman No. 52 chromatography paper were from Whatman Biochemicals Ltd, Maidstone, England. All chemicals were, where possible, analytical grade reagents.

Methylase assays

This assay measures the incorporation of radioactivity from *S*-adenosyl-L-[*Me*-^{3}H]methionine into an acid precipitable product. Standard incubation mixtures for the protein methylase initially contained 5 μmoles of Tris–HCl, pH 7.8, 1 μmole of $MgCl_2$, 10 μmoles of dithiothreitol, 0.3 nmole of *S*-adenosyl-L-[*Me*-^{3}H]methionine (approx. $5 \cdot 10^6$ cpm) and the enzyme fraction (variable amounts dependent on the specific activity) in a total volume of 0.25 ml. This incubation mixture was subsequently modified, after the establishment of the pH optimum for the protein methylase (Fig. 4), to include 5 μmoles of Tris–HCl at pH 8.4 rather than at pH 7.8. Incubation was at 37 °C for 1 h and the reaction was terminated by the addition of 0.2 ml of 0.2 M sodium pyrophosphate, 0.5 ml of 1 M $HClO_4$, and 0.1 ml of 0.5% (w/v) bovine serum albumin. The precipitated products were kept at 0 °C for 10 min, 1 ml of water was then added and the precipitate collected by centrifugation. The precipitate was dissolved in 0.3 ml of 0.2 M NaOH and reprecipitated two more times before being collected on 2.4-cm glass fiber discs. The discs were washed with 10 ml of 1 M $HClO_4$, 5 ml of water and 5 ml of 70% aqueous ethanol, subsequently dried and then counted in Aquasol in an Intertechnique liquid scintillation spectrometer. The efficiency of ^{3}H counting was approx. 25%. Controls on the reaction were standard incubation mixtures without added enzyme and the radioactivity incorporated in the controls was routinely less than 0.0001 pmole/min. All reactions were performed in duplicate and were corrected by subtraction of the control values.

The concentration of protein was determined by the method of Lowry et al. [9]. The enzymic activity was expressed as pmoles of methyl groups incorporated into an acid precipitable product per min per mg of added protein.

Polyacrylamide gel electrophoresis

Proteins were methylated as described in the previous section and the reaction products precipitated with $HClO_4$. The precipitated proteins were dissolved in 0.2 M NaOH, reprecipitated two more times and the final precipitate thoroughly washed with ethanol and ether. The precipitated proteins were then dried in vacuo. The methylated proteins were dissolved in 62.5 mM Tris–HCl, pH 6.8, 0.1 M dithiothreitol, 2% (w/v) sodium dodecylsulfate, 10% (w/v) glycerol and heated at 100 °C

for 2 min. The dissociated material was electrophoresed on a 10% polyacrylamide slab gel as described by Studier [10] and the gel then dried onto a sheet of Whatman No. 52 filter paper. The gel was sliced into 2.0-mm fractions and each fraction was dissolved in 0.1 ml of 30% (v/v) H_2O_2 by heating at 60 °C for 16 h. 4 ml of Aquasol was then added and the radioactivity in each slice quantitated by liquid scintillation counting.

Hydrolysis of methylated proteins

The methylated proteins were hydrolyzed either in 6 M HCl at 105 °C for 24 h or in 2 M NaOH at 70 °C for 16 h in evacuated, sealed tubes. The hydrolyzates were dried in vacuo and then ionophoresed on Whatman No. 52 paper at pH 2.0 [11]. The papers were stained with 0.2% (w/v) ninhydrin in acetone and then cut into 1-cm fractions. The ^3H radioactivity in each fraction was quantitated by liquid scintillation counting in Aquasol.

RESULTS AND DISCUSSION

Isolation of the protein methylase from chicken embryo nuclei

The isolation procedure is described below and the results of a representative purification from 96 9-day-old chicken embryos (starting wet weight 60 g) are summarized in Table I. All operations were performed at 0–4 °C.

TABLE I

METHYLASE ASSAYS ON FRACTIONS FROM CHICKEN EMBRYO CELLS

Approximately 50 μg of protein from each fraction was used for these assays. The assay procedure is described in Materials and Methods.

Fraction	Total protein (mg)	Specific activity*	Total activity
Whole cells	1440	0.079	113.76
Crude nuclei	232	0.164	38.05
Insoluble nuclear fraction	133	0.080	10.64
0–20% $(NH_4)_2SO_4$ fraction	24.2	0.155	3.75
20–40% $(NH_4)_2SO_4$ fraction	36.3	0.176	6.39
20–40% $(NH_4)_2SO_4$ fraction from CM-cellulose	10.1	0.379	3.83
40–60% $(NH_4)_2SO_4$ fraction	16.0	0.008	0.13

* pmoles of methyl groups incorporated into an acid insoluble product/min per mg of protein.

Chicken embryos were decapitated and thoroughly washed in 30 vol. of 0.14 M NaCl to remove as much blood as possible. The embryos were minced by extrusion through a 5-ml syringe and homogenized in a loose fitting teflon-glass tissue grinder in 9 vol. of 0.25 M sucrose, 1.8 mM $CaCl_2$. The homogenized material was filtered through two layers of cheesecloth and then 150 ml of the filtrate was layered over 150 ml of 0.34 M sucrose, 0.18 mM $CaCl_2$ for centrifugation at 600 × g for 10 min. The supernatant (the cytoplasmic fraction) was discarded. The pellets were rehomogenized in 50 ml of 0.25 M sucrose, 0.18 mM $CaCl_2$, relayered over 0.34 M sucrose, 0.18

mM $CaCl_2$ and then centrifuged as before. Resuspension and centrifugation was repeated once more and the final pellets were combined to give "crude nuclei". The red coloration of this fraction was variable and was dependent on the efficiency of washing the embryos prior to homogenization. The fraction was free of visible debris when observed in the phase-contrast microscope at 400 × magnification.

The "crude nuclei" were disrupted by blending at top speed in 100 ml of 0.14 M NaCl for 2 min in an Osterizer and were then centrifuged at 12 000 × *g* for 10 min. The pellet (the insoluble nuclear fraction) was resuspended in 10 ml of 50 mM Tris–HCl, pH 7.8, 1.4 mM β-mercaptoethanol. The supernatant was sequentially precipitated with 20, 40, and finally 60% (w/v) $(NH_4)_2SO_4$; the solid $(NH_4)_2SO_4$ was added over a 30-min period and this was followed by stirring for a further 20 min. The suspensions were centrifuged at 12 000 × *g* for 10 min and the pellets dissolved in 10 ml of 50 mM Tris–HCl, pH 7.8, 1.4 mM β-mercaptoethanol. These solutions were clarified by centrifugation and stored at 0 °C prior to the preliminary methylase assays (Table I).

The isolation of nuclei resulted in the loss of much methylating activity. The majority of this activity undoubtedly reflects the presence of cytoplasmic methylases, however, inactivation of nuclear methylases during the isolation of nuclei is a possibility. A significant amount of methylating activity was present in the "insoluble nuclear fraction". This activity was partially solubilized by sonication but not by treatment with ionic and non-ionic detergents; it was not further characterized during this study. Soluble nuclear methylases were detected only in the 0–20% and 20–40% $(NH_4)_2SO_4$ fractions. The methylase present in the 0–20% fraction was stimulated by the addition of DNA and was a DNA methylase (Greenaway, P. J., unpublished observations); the methylase in the 20–40% fraction was further characterized.

The 20–40% $(NH_4)_2SO_4$ fraction was applied to a column of CM-cellulose previously equilibrated with 50 mM Tris–HCl, pH 7.8, 1.4 mM β-mercaptoethanol and was eluted with this buffer. The effluent fractions were assayed for protein

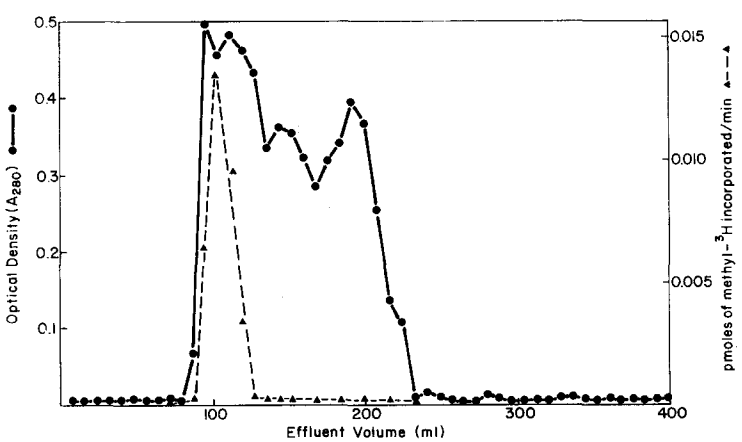

Fig. 1. Chromatography of the 20–40% $(NH_4)_2SO_4$ fraction on CM-cellulose. The 20–40% $(NH_4)_2SO_4$ fraction was applied to a column of CM-cellulose (48 cm × 2.5 cm diameter) previously equilibrated with 50 mM Tris–HCl, pH 7.8, 1.4 mM β-mercaptoethanol. Elution was with the equilibration buffer at 48 ml/h and 8-ml fractions were collected. The effluent fractions were assayed for protein ($A_{280\ nm}$) and for methylating activity.

($A_{280\ nm}$) and for methylating activity (Fig. 1). Fractions containing the methylating activity were pooled, precipitated with 45% (w/v) $(NH_4)_2SO_4$ and reassayed (Table I). Initial experiments indicated that changes in the ionic strength or pH of the eluting buffer did not remove further methylating activity from the CM-cellulose.

Chromatography on CM-cellulose resulted in an approximate 2-fold increase in the specific activity of the enzyme and in an approximate 60% recovery. The enzyme preparation obtained was still capable of endogenous methylation (methylation without added acceptor molecules) and it produced a product that was made acid soluble only by pronase digestion (Table II). It was tentatively concluded that the enzyme was a protein methylase. Analysis on sodium dodecylsulfate-polyacrylamide gels showed that the preparation was still very heterogeneous. The enzyme was reasonably stable; approx. 50% of the original activity was recovered after storage at 0 °C for 1 month.

TABLE II

THE SUSCEPTIBILITY OF THE METHYLATED PRODUCT TO ENZYMIC DIGESTION

13 μg of the 20–40% $(NH_4)_2SO_4$ fraction from CM-cellulose was incubated in the standard reaction mixture described in Materials and Methods. The methylated products were then digested with 100 μg of each enzyme listed below at 37 °C for 1 h. The digestion products were then recovered by $HClO_4$ precipitation.

Treatment	pmoles of methyl groups incorporated
1. No digestion	0.46
2. Pronase digestion	0.01
3. RNAase digestion	0.53
4. DNAase digestion	0.50

Substrate specificity and the methylated product

Polyacrylamide gels of endogenously methylated material (Fig. 2) indicated that a number of proteins with differing molecular weights were methylated. The low

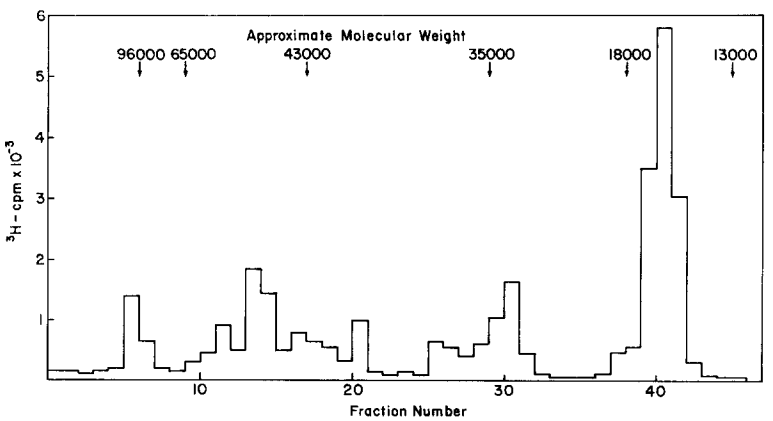

Fig. 2. Polyacrylamide gel electrophoresis of endogenously methylated proteins. 20 μg of endogenously methylated proteins were electrophoresed on a 10% polyacrylamide slab gel as described in Materials and Methods. The gel was dried onto Whatman No. 52 paper and then sliced into 2.0-mm fractions. Each fraction was dissolved in 30% H_2O_2 by heating at 60 °C for 16 h and the ^3H radioactivity present quantitated by liquid scintillation counting in Aquasol.

molecular weight proteins were the most efficient acceptors of methyl groups. Stimulation of methylation was observed when histones were added to the methylation reaction mixtures (Table III). None of the other proteins tried were able to reproduce this stimulatory effect. The possibility therefore exists that histones are the predominant in vivo methyl group acceptor molecules.

TABLE III

METHYL GROUP ACCEPTOR MOLECULES FOR THE 20–40% $(NH_4)_2SO_4$ METHYLASE

15 μg of the 20–40% $(NH_4)_2SO_4$ methylase from CM-cellulose was incubated in the standard reaction mixture described in Materials and Methods with the following acceptor molecules. The methylated products were recovered by $HClO_4$ precipitation.

Methyl group acceptor	pmoles of methyl groups incorporated
1. No added acceptor (endogenous)	0.52
2. Oxidized methylase (26 μg)*	0.52
3. Ovalbumin (50 μg)	0.49
4. Unfractionated histones (50 μg)	1.12
5. Pancreatic ribonuclease (50 μg)	0.56
6. Insulin (50 μg)	0.51
7. Bovine serum albumin (50 μg)	0.48
8. Cytochrome *c* (50 μg)	0.54
9. Lysozyme (50 μg)	0.53
10. Yeast RNA (125 μg)	0.51
11. Bacteriophage λ DNA (10 μg)	0.48

* The methylase (26 μg) was treated with 0.1 ml of performic acid (prepared by mixing 0.5 ml of H_2O_2 with 4.5 ml of 88% formic acid and used after storage at 0 °C for 1 h) at 0 °C for 4 h. The oxidized proteins were recovered by lyophilization.

The methylated product was stable to both acid and alkaline hydrolysis and cannot therefore be either carboxymethyl derivatives [3] or methylated arginine [2]. The methylated amino acid, obtained after acid hydrolysis of endogenously methylated material, co-ionophoresed with *N*-ε-methyl-L-lysine (Fig. 3) and it was therefore assumed that the enzyme methylated lysine residues. The amounts of *N*-ε-monomethyl-, *N*-ε-dimethyl- and *N*-ε-trimethyllysine was not determined. These results, together with the susceptibility of the methylated product to pronase digestion (Table II), led to the unambiguous conclusion that the 20–40% $(NH_4)_2SO_4$ fraction contained a protein methylase.

Some properties of the protein methylase

Preliminary studies on the elution of the protein methylase from Sephadex G-150 indicated that the enzyme had an approximate molecular weight of 150 000. A low ionic strength buffer was used to elute the enzyme during these experiments. Aggregation artefacts cannot therefore be excluded and thus this estimate of the molecular weight of the enzyme may be erroneously high.

The rate of protein methylation was dependent on the time of incubation at 37 °C; the initial rate decreased after 1 h and reached a plateau after incubation for 4 h. The activity was linear with respect to enzyme concentration and optimal activity in Tris–HCl buffer was observed at pH 8.4 (Fig. 4). Maximum enzymic activity was

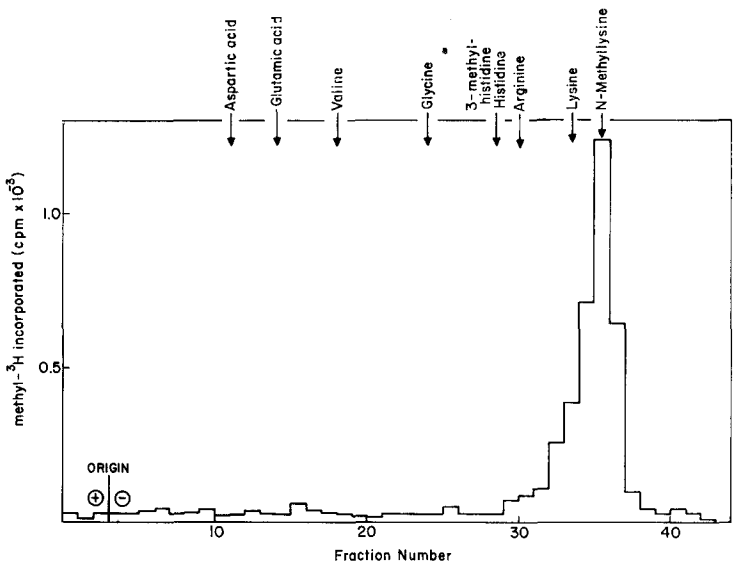

Fig. 3. Identification of the methylated amino acid. 25 μg of endogenously methylated proteins were hydrolyzed in 6 M HCl at 105 °C for 24 h. After drying in vacuo the hydrolyzate was applied to Whatman No. 52 paper for ionophoresis at pH 2.0. The paper was stained with ninhydrin and then cut into 1-cm fractions. The ^{3}H radioactivity in each fraction was quantitated by liquid scintillation counting in Aquasol.

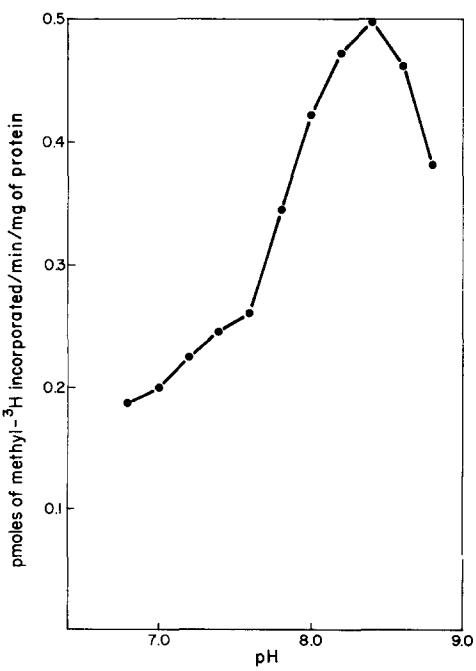

Fig. 4. The pH optimum of the protein methylase. The standard reaction mixture described in Materials and Methods, with 15 μg of the protein methylase, was used but the pH of the Tris–HCl buffer added to the reaction was varied. The methylated products were precipitated with $HClO_4$ and collected on glass fiber discs for liquid scintillation counting.

obtained with 10 μmoles of dithiothreitol and with 1 μmole of Mg^{2+}. Addition of 1 μmole of EDTA resulted in a $> 90\%$ inhibition of the enzyme reaction. Ca^{2+} replaced the requirement for Mg^{2+} but other metal cations gave increasing inhibition as follows: $Mn^{2+} < Hg^{2+} < Cu^{2+} < Pb^{2+} < Fe^{3+} < Zn^{2+} < Ni^{2+}$. Methylation was dependent on the concentration of *S*-adenosylmethionine (Fig. 5) and the enzyme showed a K_m value of $3.06 \cdot 10^{-6}$ M for this cofactor. Boiling (100 °C for 2 min) destroyed the enzymic activity and methylation was inhibited in the presence of *S*-adenosylhomocysteine and *S*-adenosylethionine.

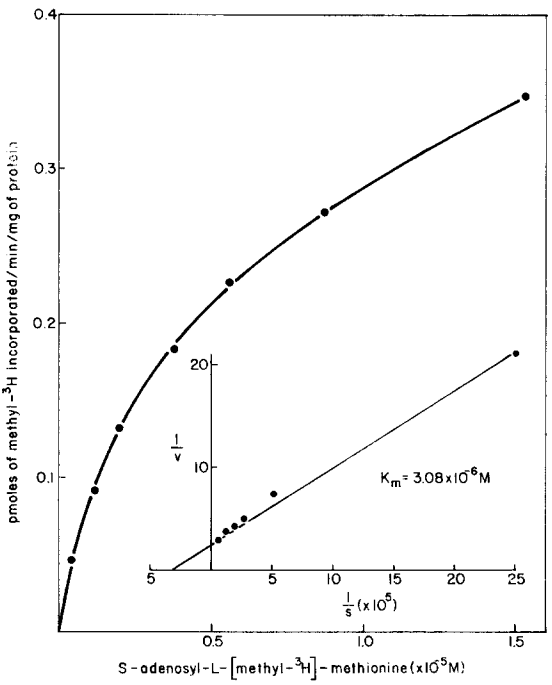

Fig. 5. The effect of *S*-adenosyl-L-methionine concentration on protein methylation. The standard incubation mixture described in Materials and Methods, with 15 μg of the protein methylase, was used; the concentration of *S*-adenosyl-L-methionine varied. The reaction products were precipitated with $HClO_4$ and collected on glass fiber discs for liquid scintillation counting.

Comparison to protein methylase III of calf-thymus nuclei

The protein methylase discussed in this paper bears much resemblance to protein methylase III isolated from calf-thymus glands [4]. Both enzymes are nuclear in origin and both show similar kinetics, including identical pH optima and K_m values for *S*-adenosylmethionine. In addition, both enzymes are stimulated by added histones and both methylate the ε-amino group of lysine residues. However, the two enzymes have different Mg^{2+} requirements; the chicken embryo methylase is optimally active with 1 μmole of Mg^{2+} whereas the calf-thymus methylase has no divalent metal requirement. More significantly and unlike the calf-thymus methylase, the chicken embryo methylase is solubilized simply by cellular disruption and it is relatively stable. The chicken embryo protein methylase is therefore more amenable to biochemical

analysis; it does, however, require further purification to free it from its endogenous methyl group acceptor.

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