J. Biochem. Mol. Biol. Vol.28, No.2 pp.149~154 March 31, 1995

Purification and Characterization of Protein Methylase II from Porcine Testis

Kikyung Jung, Myunghee Kwon, Hoi Young Lee[1], Hyang Woo Lee[2] and Sungyoul Hong*

Department of Genetic Engineering, College of Life Science and Natural Resources, Sungkyunkwan University, Suwon 440-746, [1]Laboratory of Pathology, National Cancer Institute, National Institute of Health, Bethesta, MD 20892, USA
[2]Laboratory of Biochemistry, College of Pharmacy, Sungkyunkwan University, Suwon 440-746, Korea

(Received October 15, 1994)

Abstract: Protein methylase II (S-adenosyl-L-methionine : protein O-methyl-transferase, EC 2.1.1.24; PM II) was purified approximately 1250-fold from porcine testis by fractional precipitation and DEAE-cellulose chromatography, followed by gel filtration on a Sephadex G-75 column and HPLC on a Protein Pak 125 column. The molecular weight of the enzyme was estimated to be 33,000 daltons by SDS-PAGE, which agreed with the value determined by gel filtration. Isoelectric focusing of purified PM II showed a single protein species with an isoelectric point of 6.2. The optimum pH for the reaction was 6.0. The K_m value of the enzyme was 1×10^{-5} M with a V_{max} value of 769 pmol/min/mg of enzyme. S-adenosyl-L-homocysteine is a competitive inhibitor of PM II with a K_i value of 1.38×10^{-6} M.

Key words: porcine testis, protein methylase II.

Posttranslational covalent modification of a protein is one of the most important elements to regulate its functions. One such modification is protein carboxymethylation through which a methyl group from S-adenosyl L-methionine (SAM) is transferred to carboxyl groups of acceptor proteins. Protein carboxymethylation occurs in a wide variety of prokaryotic and eukaryotic cell types (Springer *et al.*, 1979).

Protein methylase II (S-adenosyl-L-methionine: protein O-methyl-transferase, EC 2.1.1.24; PM II) was first described by Axelod and Daly (1965) as a "methanol-forming enzyme". Subsequently, this enzyme was shown to transfer the methyl group from SAM to the carboxyl side chain of proteins. This reaction results in neutralization of negative charges by the formation of protein methyl esters which undergo spontaneous and/or enzymatic hydrolysis at physiological pH and temperature. This rapid and reversible alteration of protein charge is one of the leading mechanisms which post-translationally control a number of cellular functions (Waarde, 1987).

Paik and Kim (1980) isolated an enzyme with protein methylase II activity during purification of an N-methylating enzyme. It is now clear that at least four distinct classes of protein carboxyl methyltransferases exist. The first class I, L-glutamyl methyltransferase, which retains the original E.C. number 2.1.1.24, has been identified only from chemotactic bacteria (Springer *et al.*, 1979). This enzyme class catalyzes the formation of relatively stable L-glutamic acid γ-methylesters on discrete chemoreceptor proteins. The second type of enzyme, class II D-aspartyl/L-isoaspartyl protein carboxyl methyltransfease (EC 2.1.1.77), is a ubiquitous enzyme which catalyzes the labile, methylesterification of unusual L-isoaspartyl and D-aspartyl residues in proteins. The function of this class of enzyme has been postulated to involve protein "aging and repair" (Clarke, 1975; McFadden *et al.*, 1986). The third type of enzyme, class III isoprenylcysteine protein carboxyl methyltransferase described in eukaryotes, catalyzes the methylation of a α-carboxyl groups of C-terminal cysteine residues which have been modified with a farnesyl or geranylgeranyl moiety *via* a thioester linkage. This reaction generates an unusually stable methylated product (Milburn *et al.*, 1990; Maltese *et al.*, 1989). Unlike the other classes of protein carboxyl methyltransferases, which are cytosolic, C-terminal isoprenylcysteine methyltransferase is membrane-associated in all eukayrotic cells examined to date (Hrycyna and Clarke, 1990; Stepheson and Clarke, 1990). Finally, the class

*To whom correspondence should be addressed.
Tel: 82-331-290-5853, Fax: 82-331-290-5893.
Email: syhong@yurin.skku.ac.kr

four enzyme has recently been identified in the cytosolic fraction of bovine brain and other eukaryotic cells. It also catalyzes C-terminal leucine residues on a 36 kDa cytosolic peptide (Xie and Clarke, 1993).

As cellular functions for protein carboxyl methyltransferases in eukaryotes were sought, the enzyme was thought to regulate secretion, neuronal receptor activity, celluar differentiation, leucocyte chemotaxes, and flagellar movement in sperm (O'Dea *et al.*, 1981). The control of cellular locomotion by PM II has been established in bacteria. In this case the methylation of membrane proteins by PM II regulates the direction of flagellar movement (Goy *et al.*, 1977). The role of protein carboxymethylation in flagella has also been studied in mammals. Rapid and transient increases in both protein carboxymethylation and demethylation have been reported following the addition of the specific chemoattractant f-Met-Leu-Phe to rabbit neutrophils (Pike *et al.*, 1978). This chemoattractant-stimulated locomotion was prevented by inhibiting intracellular methylation in human macrophages (Leonard *et al.*, 1978; Snyderman *et al.*, 1980).

Protein methylase II activity is increased with aging, reaching a peak near puberty in rat testis (Paik and Kim, 1971). A rapid increase in protein carboxymethylation in spermatozoa during capacitation occurs (Bardin and Gagnon, 1982; Castaneda *et al.*, 1983). However, a plausible mechanism explaining protein carboxymethylation in testis has yet to be proposed.

Although PM II is widely distributed throughout the body, its activity is especially high in testis (Paik and Kim, 1971). It was, therefore, important to determine the cellular distributions of the various components of the protein carboxymethylation system as an initial step for understanding its function. Purification and characterization of PM II should thus provide valuable information on the function of PM II in testis.

Materials and Methods

Materials

S-adenosyl-L-[methyl-^{14}C]methionine (specific activity, 50 mCi/mmol in 10% ethanol) was purchased from ICN. (Costa Mesa, USA). S-adenosyl-L-homocystein, γ-globulin, bovine serum albumin, histone type II-As, myelin basic protein, Sephadex G-75, and DEAE-cellulose were obtained from Sigma Chemical Co. (St. Louis, USA). All other reagents used were of the highest grade available.

Methods

Assay of protein methylase II: The procedure of Paik and Kim (1980) was followed with modification. Ten μl of a histone suspension (histone type II-As; 30 mg/ml) and 15 μl of enzyme preparation were added to eppendorf tubes immersed in an ice bucket containing 20 μl of a citrate-phosphate buffer cocktail, pH 6.0, (6 ml of 0.25 M citric acid, 10 ml of 0.5 M Na_2HPO_4, 8 ml of 20 mM EDTA, and 0.08 ml of 2-mercaptoethanol). After equilibration of tubes at 37℃ for 3 min, the reaction was started by adding 5 μl of [methyl-^{14}C]SAM (100 μM; 2.5 mCi/mM). The reaction was allowed to proceed for 10 min at 37℃, then stopped by addition of 0.4 ml of 15% (w/v) trichloroacetic acid (TCA). The mixture was carefully overlayered with ethanol and centrifuged at 10,000 rpm for 1 min in a tabletop microcentrifuge. The supernatant was decanted, and the precipitate was washed by repeated cycles of centrifugation and resuspention with a 15% TCA/chloroform-ether-ethanol mixture (1 : 2 : 2, v/v), and 98 % ethanol, consecutively.

Radioactivity in the protein precipitate was counted using a liquid scintillation counter with 5 ml of Bray's solution. The specific activity of protein methylase II was expressed as pmoles methyl groups transferred/min/mg of enzyme protein. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Purification of protein methylase II

Frozen, defatted porcine testis was thawed and homogenized in 4 volumes of 0.3 M sucrose solution using a Waring blender at 30 sec pulses with 1 min intervals. The homogenate was filtered through double layered cheese cloth, then centrifuged at 18,000×*g* for 30 min in a Beckman J2-21M/E high speed centrifuge (rotor: JA-20, 12,500 rpm). The resulting supernatant was centrifuged at 105,000×*g* (rotor: 50 Ti, 35, 000 rpm) for 60 min. The supernatant was brought to 70% $(NH_4)_2SO_4$ saturation (4℃) by addition of solid crystals over a period of 30 min. The precipitate was recovered by centrifugation at 39,000×*g* for 30 min, then dissolved in 32 ml of 20 mM Tris-HCl, 0.2 mM EDTA, and 15 mM 2-mercaptoethanol buffer, pH 8.0 (Buffer A), and dialyzed overnight against the same buffer using a dialysis membrane (molecular cut-off; 12,000). The dialysate (600 mg protein) was applied to a DEAE-cellulose column (3×40 cm) previously equilibrated with 1 l of Buffer A. The enzyme was eluted with a linear gradient of 0.0~0.1 M NaCl in buffer A and the column was washed with 1 M NaCl/buffer A at a flow rate of 60 ml/h. Active fractions were pooled, then concentrated by ultrafiltration using YM 10 Diaflo membrane at 30 psi for 30 min. The concentrated enzyme preparation (22.5 mg protein) was passed through a Sephadex G-75 column

(2.2×100 cm) previously equilibrated with Buffer A. Elution was performed with Buffer A at a flow rate of 30 ml/h and 4 ml fractions were collected. Fractions with PM II activity were pooled and concentrated to approximately 2 ml in an Amicon ultrafiltration cell. The protein concentration was monitored by measurement of the absorbance at 280 nm. The concentrated enzyme preparation with PM II activity (869 μg protein) was applied to an HPLC column (Protein Pak 125) equilibrated with Buffer B (20 mM Tris-HCl, pH 7.8). The flow rate was 0.5 ml/min, and the UV absorbance at 280 nm was monitored. Fractions representing absorbance peaks were collected and assayed for PM II activity.

Determination of molecular weight

SDS-polyacrylamide gel electrophoresis was performed by a modification of the method of Laemmli (1970). The active fractions of the final purification step were concentrated and diluted with an equal volume of 2 sample buffer, boiled for 5 min, then loaded onto 11% polyacrylamide slab gel (acrylamide : bisacrylamide=30 : 0.8). The samples were electrophoresed with molecular weight markers at 30 mA for 4 h. Gels were immediately stained with 0.1% Coomassie Brilliant Blue R-250 and destained with a 30% (v/v) methanol/ 10% (v/v) acetic acid mixture.

In order to estimate the molecular weight of non-denatured protein methylase II the enzyme was applied to a Sephadex G-75 column (1.2×150 cm) previously equilibrated with Buffer A. Elution was carried out with Buffer A at a flow rate of 30 ml/h. The absorbance of each fraction (2 ml) was monitored at 280 nm. In order to identify the elution position of the enzyme its activity was measured in each fraction.

Isoelectric focusing

Isoelectric focusing was performed in PhastGel IEF (pH 3 to 9) on a Phamacia LKB Phast system in order to determine the isoelectric point (pI) of purified protein methylase II. Prior to isoelectric focusing the enzyme was lyophilized and dissolved in 50 μl of water. Isoelectric focusing was performed with pI standards. Proteins in the isoelectric focusing gel were stained with coomassie brilliant blue R-250.

Results

Purification of protein methylase II

Chromatographic profiles of the purification steps were illustrated in Fig. 1 (DEAE-cellulose), Fig. 2 (Sephadex G-75), Fig. 3 (HPLC). The specific activity of

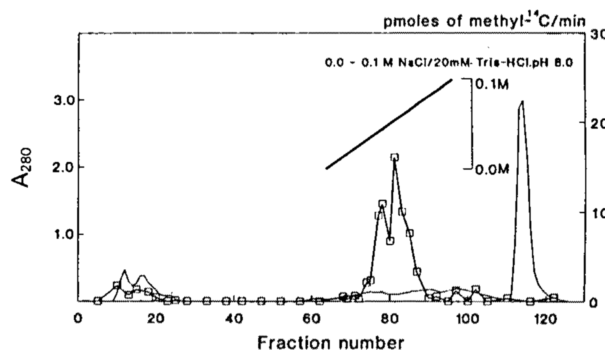

Fig. 1. Chromatography of protein methylase II on DEAE-cellulose column. 20 ml of dialysed aliquot was applied to DEAE-cellulose column (3.0×40 cm). The column was washed with 20 mM Tris-HCl, pH 8.0 containing 0.2 mM EDTA, 15 mM 2-mercaptoethanol, and eluted with a linear gradient of 0.0~0.1 M NaCl and 1 M NaCl in buffer A at a flow rate of 60 ml/h. The fractions (5 ml) were analyzed for protein (—) and for the activities of protein methylase II (□-□).

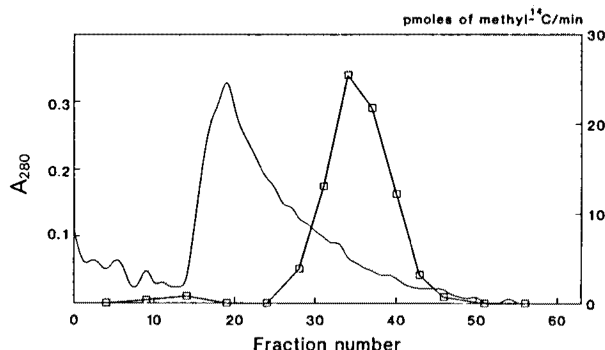

Fig. 2. Chromatography of protein methylase II on Sephadex G-75 column. The concentrated fractions (5 ml) of pooled eluate with PM II activity from DEAE-cellulose chromatography was applied to Sephadex G-75 column (2.2×100 cm) and eluted with 20 mM Tris, pH 8.0, containing 0.2 mM EDTA, 15 mM 2-mercaptoethanol, at a flow rate of 30 ml/h. The fractions (4 ml) were analyzed for protein (—) and for the activities of PM II (□-□).

purified PM II was 2,400 U, and a 1263-fold purification was achieved. The yield was 6.0% (Table 1).

Estimation of the molecular weight of protein methylase II

The major band on SDS-PAGE was estimated to be 33,000 Da (Fig. 4). The molecular weight of non-denatured protein methylase II by Sephadex G-75 chromatography was estimated to be 34,000 Da. A calibration curve for the Sephadex G-75 column was obtained by plotting the distribution coefficients (K_{av}) of the standard proteins against the logarithms of their molecular weights. The K_{av} value for protein methylase II was 0.34.

Properties of protein methylase II

Purified protein methylase II was run on PhastGel IEF. As shown in Fig. 5, the isoelectric point of porcine testis protein methylase II showed a single protein species with an isoelectric point (pI) of 6.2.

A Lineweaver-Burk plot of SAM concentration vs. the rate of methylation of histone type II-As gave a K_m value of 1.0×10^{-5} M, and a V_{max} value of 769.2 U per mg of enzyme protein. The effect of SAH on protein methylase II is shown in Fig. 6. A Lineweaver-Burk plot of methylation inhibited by SAH was of a competitive type. The K_i value for SAH was 1.38×10^{-6} M. The optimum pH for purified protein methylase II was 6.0 with methyl acceptor proteins.

Specific activities of purified protein methylase II for various protein substrates are shown in Table 2. MBP and hemoglobin were more effective than histone type II-As as a methyl acceptor.

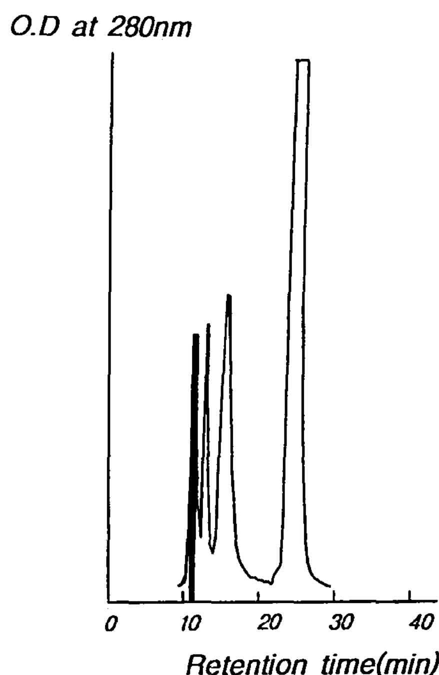

Fig. 3. HPLC of protein methylase II on Protein Pak 125 column. The concentrated fractions of pooled eluate with PM II activity from Sephadex G-75 chromatography was applied to Protein Pak 125 column (7.8×300 mm) and eluted with 20 mM Tris-HCl, pH 7.8, at a flow rate of 0.5 ml/min. The fractions (1 ml) were analyzed for protein (——) and for the activities of PM II (—).

Discussion

Protein methylase II has been purified from brain (Aswad and Deight, 1983; Kim *et al.*, 1978; Diliberto and Axelrod, 1976), thymus (Kim and Paik, 1970), and erythrocytes (Gilbert *et al.*, 1988; Kim, 1974).

Protein methylase II activity was highly localized in the cytosol of porcine testis, suggesting that the enzyme interacts with the external surface of subcellular organelles, soluble proteins, and other membrane components of the cell by neutralizing negative charges at the surface of methyl accepting proteins.

The purified enzyme preparation exhibited two bands on SDS polyacrylamide gel electrophoresis. The major band was estimated to be 33,000 Da. The major component was thought to be porcine testis PM II in accordance with the apparent molecular weight of nondenatured purified PM II obtained by Sephadex G-75 chromatography. It appears that the enzyme is a monomeric globular protein. This result is similar to a recent report on the molecular weight of PM II from *Salmonella typhimurium* (Simms *et al.*, 1987). The molecular weight of mammalian PM II enzymes was thought to be 24,000~26,000 Da in all tissues examined. However, a number of different PM II molecular weights have been reported: ox brain enzyme, 34,000 (Iqbal and Steenson, 1976); calf thymus, 35,000 (Kim and Paik, 1970); wheat germ, 41,000 (DiMaria *et al.*, 1982) Topedo electric organ, 29,000 (Haklai and Kloog, 1987), and chicken pancreas, 46,800 (Yoo *et al.*, 1991).

The isoelectric points of PM II appear to vary over a range of 4.9~6.5 depending on the tissue source, and some tissues have more than one isoelectric form

Table 1. Purification of protein methylase II from porcine testis[a].

Purification steps	Volume (ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)[b]	Purification (fold)	Yield (%)
Whole homogenate	654	24.80	1.9	31437.9	1.0	100.0
105 K Supernatant	500	17.30	3.0	25950.0	1.6	82.5
70% Amm. sulfate	150	30.00	4.8	21600.0	2.5	68.7
DEAE-cellulose	725	0.30	87.7	19067.5	46.2	60.6
Sephadex G-75	225	0.05	956.3	10743.7	503.3	34.3
HPLC	40	0.02	2400.0	1920.0	1263.2	6.0

[a] 160 g of Porcine testis was used.
[b] U: pmol of methyl-^{14}C groups incorporated to the methyl acceptor subsrate.

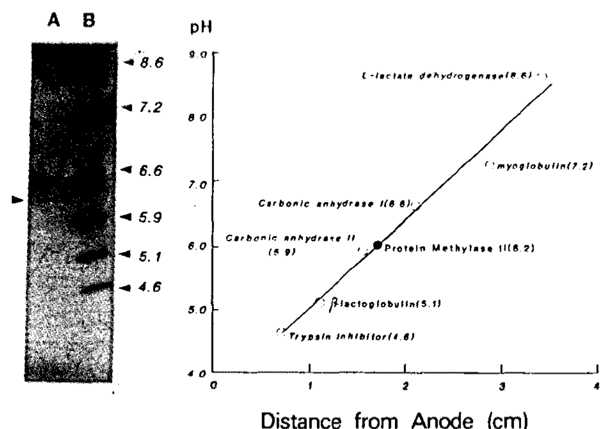

Fig. 4. SDS-PAGE (1) and calibration curve (2) of the porcine testis protein methylase II. (1) Purified protein methylase II was electrophoresed on a 1.5 mm thick slab gel containing 4.5% (stacking gel) and 11% (separating gel) acrylamide in triglycine buffer, pH 8.9. The mixture were then electrophoresed for 4 h at 30 mA. The gel was stained with coomassie blue and destained according to the method of Fair Bank *et al.* (Lane A : The molecular weight markers; Lane B : purified porcine testis protein methylase II). (2) A calibration curve for SDS-PAGE was obtained by plotting the relative mobility of the standard proteins against the logarithms of molecular weights.

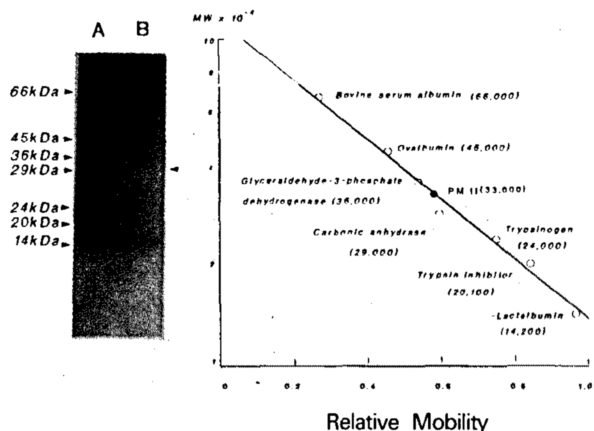

Fig. 5. Isoelectric focusing gel electrophoresis (1) and calibration curve (2). (1) Purified protein methylase II was isoelectric focusing gel electrophoresed as described in text. (Lane A : The pI standard markers, Lane 2 : the purified protein methylase II). (2) A calibration curve for isoelectric point was obtained by plotting the from anode of the standard proteins against pH.

(Aswad and Deight, 1983 ; Haklai and Kloog, 1987). However, The pI (7.4) of porcine spleen PM II (Kim *et al.*, 1994) is higher than other eukaryotic PM II values. In calf brain, Kim *et al.* (1978) found evidence of four different isoelectric forms and speculated that they might represent isozymes of PM II. The isoelectric point of porcine testis PM II was 6.2, indicating that the protein is weakly acidic.

The substrate specificities of various commercially available proteins as methyl acceptors for porcine testis PM II are shown in Table 2. Myelin basic protein and hemoglobin were more effective than histone type II-As as a methyl acceptor. The result is similar to data obtained from porcine spleen PM II. γ-Globulin is as effective as histone type II-As, while gelatin, cytochrome c, and bovine serum albumin do not have any methyl accepting capacity.

Optimum pH of mammalian PM II was varied from 6.0 to 7.5 depending on the substrate used. The optimum pH of the PM II from porcine testis was 6.0 in the presence of histone type II-As as a methyl accept-

Table 2. Substrate specificity for PM II from porcine testis.

Substrate protein[a]	Specific activity (U/mg enzyme)[b]	Relative activity
Histone type II-As (Calf thymus)	323.8	100
γ-Globulin (Bovine)	300.8	93
Myelin basic protein (Bovine brain)	608.8	188
Hemoglobin (Bovine)	470.8	145
Gelatin	–	–
Cytochrome c (Bovine heart)	–	–
Bovine albumin	–	–

[a] 0.3 mg of each substrate protein and 0.3 μg of purified enzyme were used.
[b] U : pmol of methyl-^{14}C groups incorporated to the methyl acceptor subsrate.

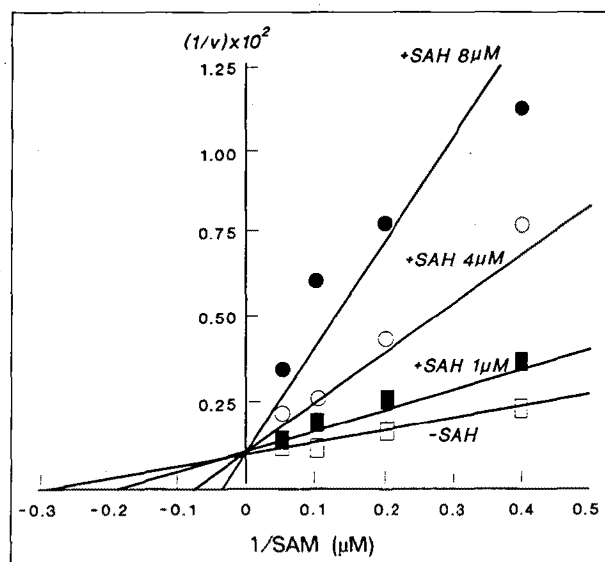

Fig. 6. Lineweaver-Burk plot of inhibition protein methylase II by S-adenosylhomocysteine. Protein methylase II activity was measured with 0.3 mg of histone type II-As as substrate at pH 6.0 in the absence and presence of S-adenosylhomocysteine as indicated. The purified protein methylase II (0.3 mg) was used as enzyme source.

ing protein.

Purified PM II has a K_m value of 1.0×10^{-5} M for SAM as a methyl donor. This value is higher than that of other mammalian PM II enzymes. However, the K_m value of porcine testis PM II for SAM was similar to that (1.7×10^{-5} M) of protein carboxyl methyltransferase from *Salmonella typhimurium*. This result is also similar to porcine spleen PM II (1.4×10^{-5} M) (Kim *et al.*, 1994).

Protein methylation reactions are generally sensitive to product inhibition by SAH, apparent inhibition constants being in the range of $0.2\sim1.5\times10^{-6}$ M (Trivedi *et al.*, 1982). Porcine testis PM II is also strongly inhibited by SAH. SAH is a competitive inhibitor of purified PM II with a K_i value of 1.38×10^{-6} M. This result is similar to K_i values of other eukaryotic PM II enzymes.

From the data now available it has been found that porcine testis PM II has many features in common with PM II enzymes from other eukaryotic tissues, in cytosolic localization, a broad substrate specificity, a high affinity for SAH, and having a monomeric form. Nevertheless, some notable differences between porcine testis PM II and other mammalian PM II enzymes were noted.

Acknowledgement

This article is offered to commemorate the retirement of professor Chung-No Joo from the Department of Biochemistry, College of Science, Yonsei University.

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