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Protein carboxyl methylation in kidney brush-border membranes

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Protein carboxyl methylation activity was detected in the cytosol and in purified brush-border membranes (BBM) from the kidney cortex. The protein carboxyl methyltransferase (PCMT) activity associated with the BBM was specific for endogenous membrane-bound protein substrates, while the cytosolic PCMT methylated exogenous substrates (ovalbumin and gelatin) as well as endogenous proteins. The apparent K_m for *S*-adenosyl-L-methionine with endogenous proteins as substrates were 30 μM and 4 μM for the cytosolic and BBM enzymes, respectively. These activities were sensitive to *S*-adenosyl-L-homocysteine, a well known competitor of methyltransferase-catalyzed reactions, but were not affected by the presence of chymostatin and E-64, two protein methylesterase inhibitors. The activity of both cytosolic and BBM PCMT was maximal at pH 7.5, while BBM-phospholipid methylation was predominant at pH 10.0. Separation of the = methylated proteins by acidic gel electrophoresis in the presence of the cationic detergent benzyldimethyl-*n*-hexadecylammonium chloride revealed distinct methyl accepting proteins in the cytosol (14, 17, 21, 27, 31, 48, 61 and 168 kDa) and in the BBM (14, 60, 66, 82, and 105 kDa). Most of the labelling was lost following electrophoresis under moderatly alkaline conditions, except for a 21 kDa protein in the cytosol and a 23 kDa protein in the BBM fraction. These results suggest the existence of two distinct PCMT in the kidney cortex: a cytosolic enzyme with low selectivity and affinity, methylating endogenous and exogenous protein substrates, and a high-affinity BBM-associated methylating activity.

Introduction

Protein carboxyl methylation is a post-translational covalent modification found in both procaryotic and eucaryotic cells [1,2]. The reversible neutralization of protein negative charges by the esterification makes protein methylation a possible regulatory agent of cellular processes. In chemotactic bacteria, for example, the specific methylation of glutamyl residues of membrane receptor proteins has been shown to play a major role in the chemotactic response [3]. A regulatory role of carboxyl methylation in eucaryotic tissues has been suggested [4,5] but strong and direct evidence is still lacking [2,6]. The eucaryotic protein carboxyl methyltransferase (PCMT) seems to be part of another, widely distributed, class of methyltransferases [2,7]. This latter class recognizes with high affinity aspartic residues that have been covalently altered by isomerization and racemization reactions to form L-iso-aspartyl and D-aspartyl residues [8]. It is proposed that the methylation of these altered residues plays a role in the metabolism of damaged proteins, at least in the brain and erythrocytes [9,10].

The proximal tubules of the kidney is the major site of reabsorption of essential metabolites from the glomerular filtrate. Preliminary studies of the protein methylation reactions occurring in the tubules have shown a unique balance between methylating and demethylating enzymes [11], raising the possibility that this methylation system may be involved in the reversible modification of some tubular functions. To further examine this possibility, we examined the presence of endogenous methyl accepting proteins located in the soluble and brush-border membrane (BBM)

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHcy, *S*-adenosyl-L-homocysteine; PCMT, protein carboxyl methyltransferase; PME, protein methyl esterase; BBM, brush-border membrane; 16-BAC, benzyldimethyl-*n*-hexadecylammonium chloride; E-64, *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine.

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fractions of the kidney cortex. We report the presence of a BBM-associated and a cytosolic PCMT which have distinct catalytic properties and substrate specificities.

Materials and Methods

Materials

S-Adenosyl-L-[*Me*-^{3}H]methionine (72.5 Ci/mmol) was obtained from New England Nuclear (Boston, MA). *S*-adenosyl-L-methionine (*p*-toluenesulfonate salt), *S*-adenosyl-L-homocysteine, gelatin, ovalbumin, bovine serum albumin, benzyldimethyl-*n*-hexadecylammonium chloride (16-BAC) and *N*-[*N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine (E-64) were purchased from Sigma (St. Louis, MO), SDS and urea (Sequanal grade) from Pierce (Rockford, IL), and reagents for electrophoresis, from Bio-Rad (Richmond, CA). All other chemicals were of the highest purity available.

Membrane isolation

Experiments were carried out on adult male Sprague-Dawley rats. The animals were killed and the kidneys quickly perfused via the abdominal aorta with a saline solution. Slices of outer cortex were cut with a razor blade and BBM were subsequently isolated by a Mg^{2+} precipitation method [12]. All preparative steps were performed in the presence of a proteinase inhibitor solution containing in final concentrations: 100 μg/ml bacitracin, 2 μg/ml aprotinin and 10 μg/ml pepstatin A. Membranes other than BBM were pelleted by centrifugation at 5000 $\times g$ for 10 min, and the BBM were obtained by centrifugation of the supernatant at 38000 $\times g$ for 20 min. The supernatant of the BBM pellet was centrifuged at 110000 $\times g$ for 60 min and was referred to as the cytosolic fraction. The pellet containing the BBM was resuspended in 50 mM mannitol, 2 mM Hepes-Tris (pH 7.5) to a concentration of about 15 mg protein/ml and stored in liquid nitrogen. The methylation activities of both BBM and cytosol were unaffected by this storage, up to 10 days (data not shown). The purity of the membrane preparation was determined by the measure of alkaline phosphatase activity and showed a mean enrichment of 10,9 $\pm$ 1.2.

Methylation reactions

Incubations were carried out at 37 °C in 100 mM Hepes-Tris buffer (pH 7.5), with 10 μM [^{3}H]AdoMet (2 μCi) and 50–100 μg protein in a final volume of 30 μl. In the experiments with exogenous protein substrates, 500 μg of the exogenous protein were added to this medium. Protein methyl esters are defined as the radioactivity that can be converted into methanol by a base treatment [13]. Briefly, the reaction was stopped by the addition of an equal volume of 1% SDS in 0.2 M NaOH to hydrolyze the radioactive methyl esters. Aliquots (40 μl) of this mixture were applied on thick filter papers that were then inserted into the necks of scintillation vials containing 10 ml of scintillation liquid (Formula 963). The capped vials were allowed to stand at room temperature for three hours to allow diffusion of the radioactive methanol produced by the hydrolysis of the methyl esters. After removal of the filters, the radioactivity transfered to the scintillation liquid was measured. The recovery of radioactivity was similar to that previously reported [14].

Methylation of BBM phospholipids was determined after a chloroform/methanol extraction of the methylated membranes. Following methylation, BBM were pelleted by centrifugation at 105000 $\times g$ for 10 min in a Beckman Airfuge. The BBM pellet was treated with 0.1 M NaOH and extracted three times with four volumes of chloroform/methanol (2:1, v/v). The organic phases were pooled, evaporated to dryness and the radioactivity was measured by liquid scintillation counting.

Acid electrophoresis

Methylated proteins were fractionated by 16-BAC acid electrophoresis on 7.5% acrylamide gels, as described by Macfarlane [13]. Following methylation, proteins were solubilized by addition of an equal volume of a stock solution containing 7% 16-BAC, 5 M urea, 10% glycerol, 50 mM acetic acid, 0.1% DTT, 2 mM EDTA and 100 μM chymostatin with 0.01% Pyronin Y as the tracking dye. Electrophoresis was carried out overnight at a constant current of 40 mA. After electrophoresis, gels were fixed in methanol/acetic acid/water (4:1:5, v/v) for at least one hour and stained for 10 min with 0.1% Coomassie blue in fixative solution. SDS-PAGE was performed using the Laemmli buffer system [15].

Fluorography was performed by impregnation with 1 M sodium salicylate acidified with 10 mM acetic acid. The gels were dried and exposed to Kodak XR-5 film for 2 to 4 weeks at −80 °C. Scanning of the fluorograms was performed by laser densitometry LKB Ultro Scan.

Results

Endogenous and exogenous PCMT activities in rat kidney cortex

The distribution of endogenous PCMT activities in the proximal cells was investigated in three fractions: cortex homogenate, cytosol and purified BBM. As shown in Fig. 1, the PCMT activity was linear up to 100 μg protein in the homogenate, while deviations from linearity were observed at 50 μg protein for both the cytosolic and BBM fractions. The specific activity of the homogenate was similar to that of the cytosolic fraction (17.5 and 15.6 pmol/mg per h), and 3-fold

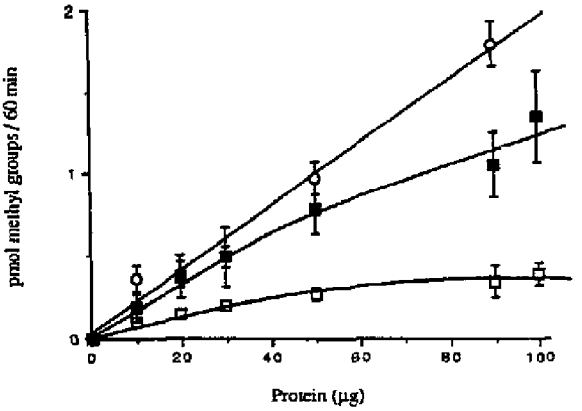

Fig. 1. Effect of protein concentration on the rate of endogenous protein carboxyl methyltransferase activities in kidney homogenate (○), cytosolic (■) and brush-border membranes (□) fractions. PCMT activities were assayed as described in Materials and Methods. Each value represents the mean ± S.D. of three different experiments carried out in triplicate.

higher than for the purified BBM (5.1 pmol/mg per h). Total endogenous activity present in the BBM fraction was 2.6% of the activity found in the homogenate. This percentage was similar to that of alkaline phosphatase, a marker enzyme specific for BBM [12].

Kinetic analysis of endogenous PCMT activities in the cytosol and BBM, with their respective endogenous proteins as substrates, revealed very distinct kinetic parameters toward AdoMet (Fig. 2). The apparent maximal velocity was 10-fold higher for the cytosolic enzyme (150 vs. 17 pmol/mg per h) while the apparent K_m for AdoMet was much lower in the BBM (4 μM vs. 30 μM).

TABLE I

Specific activities of PCMT for endogenous and exogenous substrates in kidney fractions

Fractions (50 μg protein) were incubated 10 min with or without 500 μg exogenous substrates and the methylation was measured with the methanol diffusion assay. Values for endogenous methylation were substracted to give the exogenous methylation values. The values are means ± S.D. of three distinct experiments.

Fractions	Methyl groups incorporated (pmol/mg protein per 10 min)			
	endogenous methylation	exogenous methylation		
		gelatin	ovalbumin	BSA
Homogenate	6 ± 2	80 ± 5	28 ± 5	3 ± 1
Cytosol	4.9 ± 0.9	69 ± 7	43 ± 2	0.9 ± 0.1
BBM	1.5 ± 0.9	2 ± 1	0.02 ± 0.06	0.1 ± 0.2

Table 1 shows the PCMT activities towards three exogenous substrates (gelatin, ovalbumin and bovine serum albumin (BSA)) in the homogenate, cytosol and membranes. Addition of gelatin caused 13- and 14-fold stimulations of the PCMT activities associated with the homogenate and cytosolic fractions. Ovalbumin caused 4.6- and 8.8-fold stimulations while BSA was not a good substrate with 0.5- and 0.18-fold stimulations, for the same fractions.

None of these exogenous proteins was a good substrate for the PCMT associated with the BBM (Table I). The rate of methylation of gelatin was strongly dependent on the amount of soluble proteins, while addition of a greater amount of membrane proteins in the assay did not produce any increase of BBM methylation activity (Fig. 3). Membrane disruption by solubi-

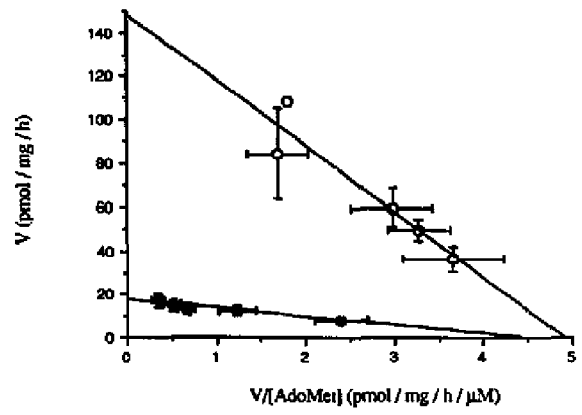

Fig. 2. Eadie-Hofstee plot of the endogenous protein carboxyl methylation activities for the cytosol (○) and BBM (●) fractions. 50 μg of cytosolic or BBM proteins were incubated with [^{3}H]AdoMet (1.5–60 μM) (3 μCi) and the methylation measured as described. Slopes were determined by linear regression from data obtained from four experiments carried in quadruplicate.

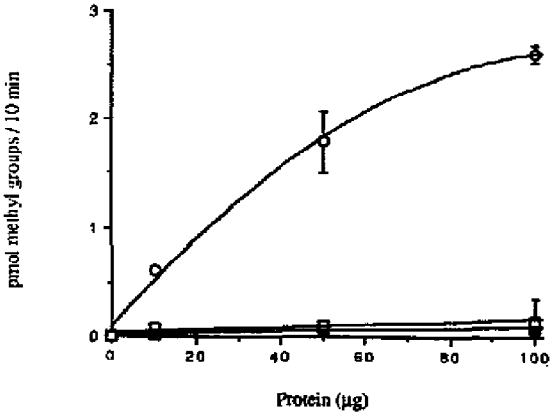

Fig. 3. Effect of protein concentration on the methylation of gelatin by brush-border membranes in the presence (□) or absence (■) of Triton X-100 and by the cytosolic fraction (○). Fractions were incubated in the presence of 500 μg gelatin and methylation was measured as described in Materials and Methods. The values are the means ± S.D. of three experiments.

lization of the BBM with Triton X-100 had no effect on the methylation of gelatin (Fig. 3), suggesting that the absence of recognition of the exogenous substrates by the membrane-bound enzyme was related to the properties of the enzyme and not to reduced accessibility of the substrates to the catalytic sites on the membrane itself or in the intravesicular space.

Effect of the incubation pH on BBM-associated endogenous PCMT activity

Fig. 4 shows the effect of pH on the endogenous PCMT activities of the cytosol and BBM. Both activities showed a similar pH-dependence, with a maximum at pH 7.5. Increasing the pH greatly reduced the activity of base-labile protein methylation for both fractions, but resulted in an increase of base-stable and chloroform extractable methylated products in the BBM (Fig. 4). The incorporation of [^{3}H]AdoMet into these base-stable linkages was maximal at pH 10.0 and corresponds to the formation of methylated phospholipids [16]. These data thus suggest the simultaneous presence of both protein and phospholipid methylation activities in kidney BBM. The distinct chemical properties of the phospholipid methylation system rules out any significant interference with the measurement of PCMT activity.

Modulation of kidney PCMT activity by AdoHcy and PME inhibitors

Methyltransferase activities from the cytosol and BBM were also studied following addition of inhibitors of transferase and esterase reactions (Table II). The activities of both fractions were inhibited to the same extent (60%) by 100 μM AdoHcy, a well known competitive inhibitor of methyltransferases [17]. Proteinase inhibitors with C-terminal aldehydes such as chymostatin and E-64 are very active as protein methylesterase (PME) inhibitors [18]. They had no effect on the methylation activities of the cytosolic and BBM enzymes suggesting that the esterase activity did not interfere with the methyltransferases.

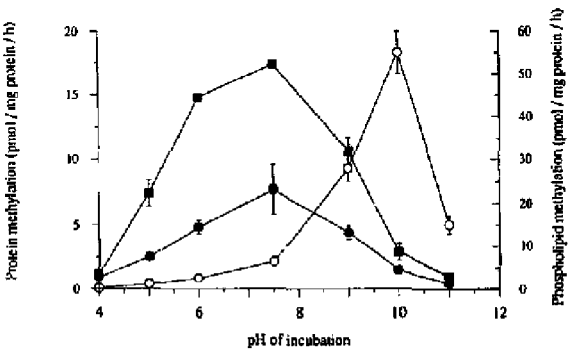

Fig. 4. Effect of pH on the rate of methylation of endogenous proteins of the cytosol (■) and BBM (●). Proteins (50 μg) were incubated in sodium phosphate (pH 4.0), Mes-Tris (pH 5.0 and 6.0), Hepes-Tris (pH 7.5), CHES-NaOH (pH 9.0), Tris-CAPS (pH 10.0) or CAPS-KOH (pH 11.0) buffers for 60 min at 37°C and the extent of methylation measured as described. Phospholipid methylation was estimated by measuring the amount of radioactivity extracted from base-treated BBM with chloroform/methanol (2:1, v/v) (○). The values are means ± S.D. of three experiments.

TABLE II

Effect of AdoHcy and PME inhibitors on the methylation of endogenous proteins

Incubation of cytosolic and BBM proteins (50 μg) was carried out as described with the presence of AdoHcy, chymostatin or E-64 in the media. Reactions were started by the addition of the proteins and the radioactivity incorporated as radioactive methanol measured as already described. Values are means ± S.D. of three different experiments.

Fractions	Methyl groups incorporated (pmol/mg protein per 60 min)			
	control	100 μM AdoHcy	50 μM chymostatin	5 μM E-64
Cytosol	16 ±3	9 ±2	15.7±0.2	13.7±0.6
BBM	5.5±0.9	2.2±0.5	5.9±0.4	6 ±2

Identification of methyl-accepting proteins in cytosolic and BBM fractions by 16-BAC electrophoresis

In order to further distinguish the BBM-associated PCMT from the cytosolic PCMT activity, the endogenous methyl accepting proteins from both fractions were separated by acid electrophoresis performed in the presence of a cationic detergent (16-BAC) [13].

The methyl accepting proteins present in the cytosolic and brush-border membranes fractions were revealed by fluorography, and the resulting fluorograms were scanned by laser densitometry for accurate comparison of the labelling patterns (Fig. 5). Eight major (14, 17, 21, 27, 31, 48, 61 and 168 kDa) proteins were methylated in the cytosol (Fig. 5A), while five major polypeptides (14, 60, 66, 82, and 105 kDa) were methylated in the BBM fraction (Fig. 5B). We found no correlation between the amount of label and the intensity of the protein stain. For example, actin (45 kDa), which is one of the major proteins found in BBM, was not significantly methylated. Incubation of the BBM in the presence of cytosol did not increase the radiolabelling of any of the membrane methyl accepting proteins (not shown), thereby suggesting that these substrates were specifically recognized by the BBM enzyme. Addition of 100 μM AdoHcy (Fig. 5, dashed lines) greatly reduced the amount of radioactivity associated with methyl accepting proteins of the cytosol and BBM, indicating the enzymatic nature of the transfer of a methyl group from AdoMet to these protein substrates.

The base-lability of the methyl esters was evaluated by separation of the methylated proteins from both

cytosol and BBM by SDS-PAGE, since most of the eukaryotic methyl esters are rapidly hydrolyzed under the alkaline conditions used in this type of gel [19]. As shown in Fig. 6, the moderately alkaline conditions almost completly abolished the labelling of cytosolic and BBM proteins, except for a 21 kDa protein in the cytosol and a 23 kDa protein in the BBM. These data thus support the idea that most of the methylated proteins of both fractions, being sensitive to alkaline hydrolysis, are carboxylmethylated.

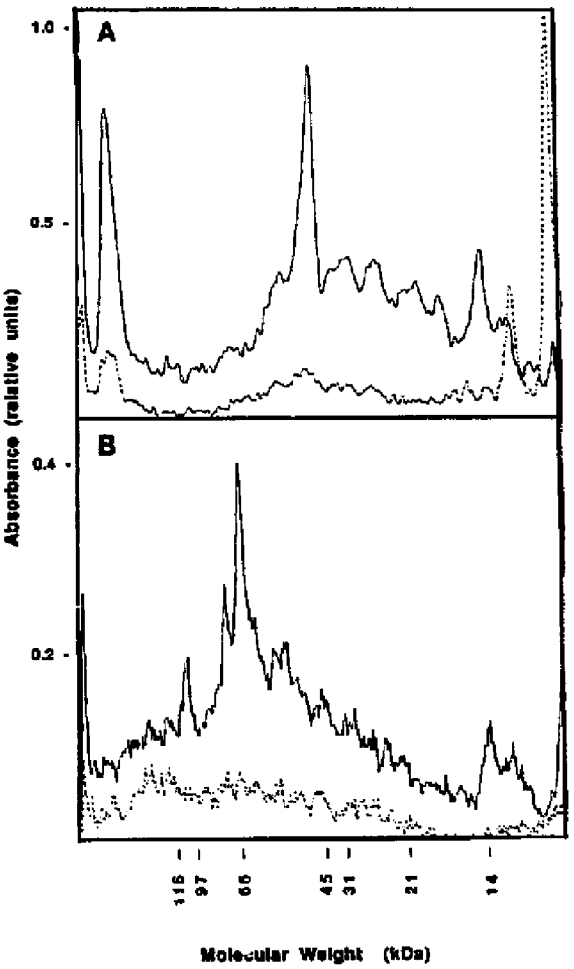

Fig. 5. Densitometric scanning of the cytosolic and BBM methylated proteins separated by acid electrophoresis. Cytosolic (A) or BBM (B) proteins (100 μg) were methylated by incubation with 10 μM [^{3}H]AdoMet (5 μCi) at pH 7.5. Following incubation, the proteins were solubilized by the addition of an equal volume of sample buffer containing 7% 16-BAC, 5 M urea, 10% glycerol, 0.1% DTT and 2 mM EDTA and applied onto a 7.5% acrylamide gel. Radioactivity was detected by fluorography, and the resulting fluorograms scanned by laser densitometry. Dashed lines represent the methylation patterns obtained in the presence of 100 μM AdoHcy. The molecular mass standards were β-galactosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa).

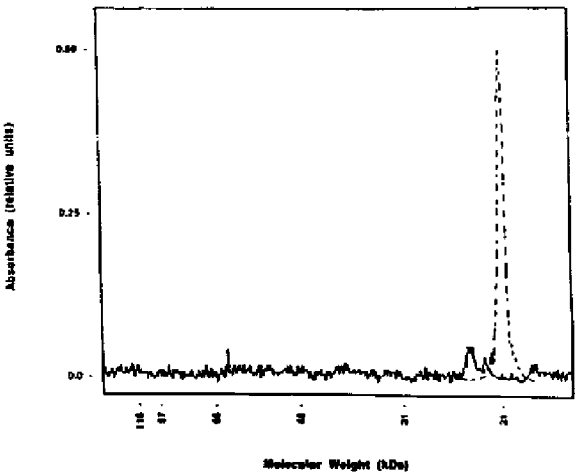

Fig. 6. Densitometric scanning of the cytosolic and BBM methylated proteins separated by SDS-PAGE. Cytosolic (dashed line) or BBM proteins (100 μg) were methylated by incubation with 10μM [^{3}H]AdoMet (5 μCi) at pH 7.5. Following incubation, the proteins were solubilized by the addition of an equal volume of Laemmli solubilization buffer and applied onto a 11% acrylamide gel. Radioactivity was detected by fluorography, and the resulting fluorograms scanned by laser densitometry. The molecular weight standards are listed in Fig. 5.

Discussion

In this paper, we present evidence for the existence of two protein methyltranferase activities localized, respectively, in the soluble fraction and in the brush-border membranes of the kidney cortex. These carboxyl methyltransferase activities were demonstrated according to three criteria: (i) the incorporation of radioactivity in both fractions is base-labile, a well established feature of eucaryotic protein methyl esters [19], (ii) the amount of methyl esters formed is greatly reduced by concomitant incubation with AdoHcy, which acts as a competitive inhibitor of methyltransferase-catalyzed reactions [17], and (iii) specific endogenous substrates can be identified following acid gel electrophoresis.

The BBM-associated protein methylation activity was easily distinguished from phospholipid methylation which was maximal at pH 10 and produced base stable and chloroform-extractable methylated lipids (Fig. 4). A similar phospholipid methylation system has been described in rat small-intestinal BBM [16]. The simultaneous presence of both protein and lipid methylation at neutral and alkaline pH, respectively, was also reported in rat colonic basolateral membranes [20].

The PCMT activities of both cytosol and BBM were not affected by the presence of protein methyl esterase

(PME) inhibitors (Table II). This enzyme, which catalyze the hydrolysis of ovalbumin methyl esters, has been found to be predominantly located in the kidney cortex proximal tubules [21]. The lack of effect of PME inhibitors may indicate that either the methylesterases present in the kidney cortex do not recognize the methylated endogenous proteins as substrates or that the experimental conditions used were not adequate for the expression of the esterase activity. For example, the cytosolic PCMT incorporated 0.8 pmol of methyl groups into protein substrates under our assay conditions (50 μg protein, 60 min incubation) which make a total concentration of 0.03 μM methyl esters; this value is much lower than the estimated K_m of the kidney methylesterase (5.9 μM for ovalbumin methyl esters) [22]. It is thus possible that the affinity of the methylesterase for the methylated proteins was too low for the activity to be detected.

PCMT from a wide variety of tissues have been shown to exist as cytosolic enzymes [2], although in some cases PCMT activity has been detected in membranes [20]. Here we show that the kidney cortex possesses two distinct PCMT activities, one soluble and one membrane-bound, and that these activities present many different characteristics. The first difference resides in their clearly distinct kinetic parameters towards AdoMet with their respective endogenous proteins as substrates. The cytosolic PCMT was found to be a low-affinity, high-capacity enzyme, whereas the BBM-associated PCMT seems to possess a relatively low methylation capacity with high affinity toward its membrane-bound substrates. The second difference concerns their different activities toward exogenous substrates such as ovalbumin and gelatin. The cytosolic PCMT activity was greatly enhanced by these protein substrates. On the contrary, under the assay conditions used in this study, the BBM enzyme showed a lack of recognition for these same substrates. This property of the kidney BBM enzyme is also very different from the high reactivity of the rat colonic basolateral membrane enzyme towards BSA [20]. Thirdly, distinct endogenous methylated proteins located in the cytosol and in the BBM could be revealed by the acidic gel conditions used in our study. The cytosol fraction contained a number of endogenous methyl-accepting proteins with a 48 and 168 kDa polypeptides as the best substrates, while the BBM possess intrinsic methyl accepting proteins of 60, 66, 82 and 105 kDa. We found that over 98% of the radiolabelling of either the kidney cytosolic or BBM proteins disappeared after electrophoresis on alkaline SDS-polyacrylamide gels [Fig. 6]. The base-lability of the methyl esters described here is thus consistent with the methylation of β-aspartyl or L-isoaspartyl residues [19] and thus may be associated with class II PCMT. The base-resistant, low molecular weight methylated proteins present in the cytosol and in the BBM may correspond to C-terminal carboxylmethylation catalyzed by a distinct class of methyltransferase [23].

Class II PCMT, which recognize a wide variety of exogenous substrates, has been associated with a D-aspartyl/L-isoaspartyl methyltransferase activity and with a possible role in the metabolism of damaged proteins [8–10]. The soluble enzyme studied here, which presents many characteristics of class II PCMT, is thus probably involved in a similar function. The membrane-bound enzyme, however, may play a distinct physiological role. Recently, a novel type of enzyme that catalyzes the base-stable methylation of the α-carboxyl group of C-terminal cysteine residues has been described [23–25] and in some cases these enzymes have been shown to exist as membrane-bound species [26–28]. The possible GTP-dependence of the carboxyl methylation catalyzed by these enzymes [29] may also suggest that methylation may be altered by hormonal stimulation of cells. However, it is not clear at this point whether the BBM-associated enzyme represents a member of this family, since methylation by this latter class results in base-stable α-carboxyl methyl esters [23,24], while most of the methyl esters obtained with the BBM enzyme were highly base-sensitive. Examination of the modulation of its activity by stimulatory ligands and use of farnesylated synthetic peptides [27] should provide new insights into the determination of the functional role of this enzyme. The brush border membrane of kidney proximal cells is a major site for the active transport of essential metabolites present in the glomerular filtrate and is under close hormonal regulation [30]. The preferential localization of a BBM-associated protein carboxyl methyltransferase with the capacity of neutralizing negative charges on specific membrane proteins may thus have important consequences on the various functions played by this membrane.

Acknowledgements

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