Asymmetrical distribution of L-isoaspartyl protein carboxyl methyltransferases in the plasma membranes of rat kidney cortex

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We have studied the distribution of membrane-associated L-isoaspartyl protein carboxyl methyltransferases (PCMTs) in plasma membranes purified from rat kidney cortex. Addition of CHAPS to brush-border membranes (BBM) and basolateral membranes (BLM) was required to measure optimal membrane-dependent methylation of ovalbumin and TS-isoD-YSKY, substrates of L-isoaspartyl PCMTs. Extraction of both membrane-associated enzymes was achieved with detergents, but not with high-salt solutions, suggesting a strong membrane attachment. However, upon phase partitioning using Triton X-114, both enzymes were predominantly associated with the detergent-poor phase, suggesting a relatively hydrophilic nature. The enzymes showed similar catalytic properties such as substrate recognition and affinity towards the methyl donor, S-adenosyl-L-methionine. The activity of the BBM enzyme, however, was about 2-fold higher than that of the BLM enzyme. Identification of the

endogenous substrates located in the two plasma membranes by acidic gel electrophoresis in the presence of a cationic detergent revealed significant differences in the methyl-accepting proteins of both membranes. The BBM-methylated proteins had sizes of 35, 50 and 54 kDa, whereas the major BLM-methylated substrates were of 97 and 100 kDa. The enzymes showed distinct behaviour on Mono Q anion-exchange chromatography. The BBM-associated PCMT did not bind to the column, being eluted in the flow-through, whereas the BLM enzyme bound to the column and was eluted at 0.15 M NaCl. Moreover, the two enzymes had different molecular masses under both denaturing and nondenaturing conditions, the BLM PCMT migrating at an apparent molecular mass of 29 kDa, compared with 27 kDa for the BBM enzyme. Taken together, these results show the presence of two distinct L-isoaspartyl PCMTs in the plasma membranes of the kidney cortex.

INTRODUCTION

L-Isoaspartyl/D-aspartyl protein carboxyl methyltransferases (PCMTs) (EC 2.1.1.77) are widely distributed enzymes that transfer a methyl group from S-adenosyl-L-methionine (AdoMet) to the carboxyl group of D-aspartyl and L-isoaspartyl residues in proteins (Ota and Clarke, 1990; Barten and O'Dea, 1990). These residues arise primarily from the spontaneous deamidation of asparagine residues which, via a succinimide intermediate, yields racemized or isomerized D-aspartyl and L-isoaspartyl residues (Geiger and Clarke, 1987; Stephenson and Clarke, 1989). The exact physiological function of the PCMT-catalysed methylation of these altered residues has not been determined precisely, but several studies have suggested that methylation may represent a key reaction in the repair of the damaged proteins. For example, incubation of L-isoaspartyl-containing peptides with AdoMet and purified PCMT results in a 80% conversion of the altered residues into normal aspartate (Johnson et al., 1987a), and methylation of deamidation-damaged calmodulin restores a substantial proportion of its biological activity (Johnson et al., 1987b).

Most of the knowledge on the structural and functional properties of L-isoaspartyl PCMT has been derived from studies on the soluble forms of these enzymes. PCMTs have been purified from a variety of tissues (Ota and Clarke, 1990), and a number of isoforms have been detected by anion-exchange chromatography or isoelectric focusing (Cusan et al., 1981; Aswad and Deight, 1983; Ota et al., 1988). The isoenzymes appear very similar in their kinetic properties and share a high degree of sequence similarity (MacLaren et al., 1992; Potter et

al., 1992). The physiological significance of the presence of these isoforms is yet to be determined, but seems to arise from the alternative splicing of a single PCMT gene (MacLaren et al., 1992; Romanik et al., 1992).

A number of reports describing membrane-associated PCMTs have appeared over the years (Iqbal and Steenson, 1976; Brown, 1984; Sellinger et al., 1987; Saïdo et al., 1987; Gingras et al., 1991). However, these enzymes have not been characterized in great detail. We have recently been successful in purifying the brush-border-membrane (BBM)-associated PCMT from rat kidney cortex (Boivin et al., 1993). The purified enzyme showed very similar structural and kinetic properties to known cytosolic methyltransferases, suggesting that the membrane form of these enzymes may play a physiological role similar to that played by the cytosolic PCMT. It is thus possible that membrane-associated PCMTs would methylate damaged proteins not accessible to the cytosolic enzyme *in vivo*.

In this paper, we report the identification of another membrane-associated form of this class of enzymes in the basolateral plasma membrane of kidney cortex. The results presented here provide evidence that the enzyme located in this membrane is distinct from the BBM-associated enzyme, and thus suggest an asymmetrical localization of these enzymes within polarized cells.

MATERIALS AND METHODS

Materials

S-Adenosyl-L-[methyl-³H]methionine ([³H]AdoMet; 73 Ci/mmol) was from DuPont-New England Nuclear (Boston, MA,

Abbreviations used: AdoMet, S-adenosyl-L-methionine; BBM, brush-border membranes; BLM, basolateral membranes; γ -GT, γ -glutamyl transpeptidase; PCMT, protein carboxyl methyltransferase; 16-BAC, benzyldimethyl-n-hexadecylammonium chloride.

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U.S.A.). 16-Benzyltrimethyl-n-hexadecylammonium chloride (16-BAC) was purchased from Aldrich. Superdex 75 and Mono Q HR 5/5 chromatography columns and Percoll were from Pharmacia (Montréal, Canada). The synthetic peptide TS-isoD-YSKY was kindly provided by Dr. R. Stephenson and Dr. S. Clarke (University of California, Los Angeles).

Membrane isolation

BBM were purified from rat kidney cortex by a Mg^{2+} precipitation method (Booth and Kenny, 1974). The final pellet was resuspended in 50 mM mannitol/5 mM Hepes/Tris (pH 7.5) at a protein concentration of 15 mg/ml and stored in liquid N_2 until use. Basolateral membranes (BLM) were purified with a self-orienting Percoll gradient method, as described previously (Gingras et al., 1993). After elimination of Percoll by centrifugation for 1 h at 110000 g, the membranes were resuspended in the mannitol buffer at a protein concentration of 15 mg/ml and stored in liquid N_2 . Storage of the membranes under these conditions had essentially no effect on methylation activities, up to 1 month. γ -Glutamyl transpeptidase (γ -GT; BBM marker) and Na^+/K^+ -ATPase (BLM marker) enrichments were measured routinely as quality controls for the preparations (Gingras et al., 1993). For purified BBM, enrichments for γ -GT and Na^+/K^+ -ATPase were 11.6 ± 1.2 and 1.1 ± 0.1 respectively, whereas enrichments in purified BLM were 0.96 ± 0.02 and 16.5 ± 1.9 .

Membrane solubilization

Membranes were diluted to 10 mg of protein/ml in 20 mM Tris/HCl (pH 8.0)/1 mM EDTA/0.5 % (w/v) CHAPS and kept on ice for 1 h. Solubilized material was separated from residual membranes by centrifugation in a Beckman Airfuge. Under these conditions, up to 80 % of BBM and 75 % of BLM proteins were solubilized.

Column chromatography

Solubilized membranes (1 ml, 7–8 mg of protein) were applied to a Superdex 75 f.p.l.c. column equilibrated with 20 mM Tris/HCl (pH 8.0)/0.05 % CHAPS (Buffer A). Protein were eluted at a flow rate of 0.5 ml/min, and 0.25 ml fractions were collected. The activity of each fraction was measured by addition of 20 μ l of the fractions to the methylation incubation medium, as described below. The fractions containing PCMT activity were pooled and concentrated by dialysis against 20 % (w/v) poly(ethylene glycol) (8000 Da) in Buffer A. At this stage, both PCMTs were enriched 11-fold. The resulting pools were applied to a 1 ml f.p.l.c. Mono Q column equilibrated with Buffer A. Proteins were eluted at a flow rate of 0.7 ml/min, with an exponential NaCl gradient (0–1 M NaCl), and 0.5 ml fractions were collected.

Methylation reactions

Proteins or column fractions were incubated in 100 mM Hepes/Tris (pH 7.5) with 2 μ M [3H]AdoMet (2 μ Ci) and 360 μ M ovalbumin or 4 μ M TS-isoD-YSKY for 60 min at 37 °C. The reaction was quenched by addition of 1 vol. of 1 % SDS in 0.2 M NaOH, and portions of this mixture were applied to thick filter papers pleated into accordions. The filter papers were then

inserted in the neck of scintillation vials containing 10 ml of liquid-scintillation-counting fluid (Formula 963; DuPont-NEN). The vials were capped and left for 2 h to allow the radioactive methanol formed by base-catalysed hydrolysis of the methyl esters to diffuse into the organic phase. Controls (no protein added) were performed in parallel and used as blanks.

Acidic gel electrophoresis

The discontinuous buffer system of MacFarlane (1983) was used. Methylation reactions were stopped with 1 vol. of 10 % (w/v) 16-BAC/5 M urea/10 % (w/v) glycerol/2 mM EDTA/2 mM dithiothreitol plus 0.01 % (w/v) Pyronin Y as the tracking dye, and the solubilized proteins were applied to 7.5 %-acrylamide gels. Electrophoresis was carried out overnight at a constant current of 30 mA. Gels were fixed in 40 % (v/v) methanol/10 % (v/v) acetic acid for 3 h with 4 changes of fixative solution to remove excess 16-BAC, and stained for 15 min with 0.1 % (w/v) Coomassie Blue in the fixative solution. After destaining, gels were impregnated with 1 M sodium salicylate acidified with 10 mM acetic acid and dried under low heat by using a high-vacuum pump. The dried gels were exposed to Kodak XAR films at -70 °C for at least 1 month.

SDS/PAGE

SDS/PAGE was carried out in the Laemmli (1970) buffer system, with an LKB 2050 Midget electrophoresis unit. Samples were applied, without boiling, to 12.5 %-polyacrylamide gels. Electrophoresis was carried out for 90 min at a constant voltage of 100 V. The resulting gel was carefully sliced in 1 mm sections, which were then placed in 1.5 ml polypropylene tubes containing 200 μ l of 1 % (w/v) Triton X-100/50 mM Hepes/Tris (pH 7.5)/15 mM β -mercaptoethanol. Proteins were eluted from the gel sections by overnight shaking of the tubes at room temperature. The L-isoaspartyl PCMT activity was measured by incubating 20 μ l portions of the mixtures at 37 °C for 90 min in 100 mM Hepes/Tris (pH 7.5), containing 2 μ M [3H]AdoMet and 360 μ M ovalbumin.

Triton X-114 phase partitioning

Membranes were solubilized with 2 % (w/v) Triton X-114 for 20 min on ice and the solubilized material was collected by ultracentrifugation. Then 200 μ l of 0.25 M sucrose was carefully applied on top of the solubilized material, and the phase separation of the detergent was performed by placing the tubes at 30 °C for 5 min. The upper (detergent-poor fraction) and lower (detergent-rich fraction) phases were carefully aspirated, and the methylation activity located in both fractions was measured as described above.

RESULTS

Association of L-isoaspartyl PCMT activities with kidney cortex plasma membranes

L-Isoaspartyl PCMT activities associated with the purified BBM and BLM fractions were identified by incubation of the membranes with a highly specific substrate for this class of enzymes, the synthetic peptide TS-isoD-YSKY, which is derived from an isoaspartyl-containing sequence of glucagon (Ota et al., 1987). Addition of 0.5 % CHAPS strongly stimulated the methylation of this substrate: an 8-fold increase in the initial rate of methylation of TS-isoD-YSKY (30 versus 4 pmol/min per mg)

Figure 1 Effect of CHAPS on the initial rate of methylation of TS-isoD-YSKY

Methylation of the synthetic isopeptide TS-isoD-YSKY by BBM (●, ○) and BLM (■, □) membrane proteins (50 μ g) was carried out at 37 °C in 100 mM Hepes/Tris (pH 7.5) with 2 μ M [3 H]AdoMet and 4 μ M of the peptide in the presence (●, ■) or absence (○, □) of 0.5% CHAPS. The methylation activities were quantified by measuring the radioactive methanol produced by base treatment, as described in detail in the Materials and methods section. Values represent the means of data obtained from three distinct membrane preparations.

Table 1 Release of L-isoaspartyl PCMTs from kidney cortex plasma membranes

Kidney cortex BBM and BLM were diluted to 10 mg of protein/ml in extraction media containing 10 mM Tris/HCl (pH 8.0) with or without 0.5% CHAPS and 1 M KCl, and the membranes were incubated with shaking for 1 h at 4 °C. The solubilized proteins were separated from the residual membrane proteins by centrifugation at 100 000 g for 30 min, and methylation activity associated with the supernatant was measured as described in the legend of Figure 1, with 360 μ M ovalbumin as the methyl acceptor. The activity recovered in the supernatant is expressed as a percentage of the total activity measured in the presence of 0.5% CHAPS. The results are means $\pm$ S.D. from three experiments.

Treatment	L-Isoaspartyl PCMT activity in supernatant (% of total activity)	
	BBM	BLM
No addition	0.8 $\pm$ 0.5	1 $\pm$ 0.3
1 M KCl	5 $\pm$ 1	8 $\pm$ 1
0.5% CHAPS	82 $\pm$ 6	79 $\pm$ 8
0.5% CHAPS + 100 mM KCl	85 $\pm$ 7	83 $\pm$ 4

was observed with BBM, whereas a 2-fold increase was observed with BLM (12 versus 6 pmol/min per mg) (Figure 1). CHAPS was very effective in extracting the membrane-bound enzymes from their native membranes. As shown in Table 1, 80% of the PCMT activity of both BLM and BBM could be extracted with CHAPS (0.5%), whereas incubation of the plasma membranes with 1 M KCl did not result in a significant solubilization of these enzymatic activities. Thus the membrane-associated L-isoaspartyl PCMTs appear to be strongly associated with the membranes.

Triton X-114 phase partitioning (Bordier, 1981) was used to characterize further the degree of association of the enzymes with their respective membrane. PCMT was solubilized efficiently from either membrane (79% solubilization) (Table 2). Upon

Table 2 Triton X-114 phase separation of kidney cortex plasma-membrane L-isoaspartyl PCMTs

Kidney cortex plasma membranes (BBM and BLM) (2 mg of protein) were diluted to 1.0 ml in 2.0% (w/v) Triton X-114/20 mM NaCl/10 mM Tris/HCl, pH 7.5, and the membranes were incubated with shaking for 20 min at 4 °C. The non-solubilized material was sedimented by centrifugation at 100 000 g for 30 min. The solubilized proteins were subjected to phase separation at 30 °C for 5 min, as described by Bordier (1981). The detergent-rich and detergent-poor phases were separated through a sucrose cushion by centrifugation at 3000 g for 5 min and assayed for methylation activity and protein. The results are means $\pm$ S.D. from three experiments.

Fraction	PCMT activity			
	BBM	(% of total activity)	BLM	(% of total activity)
	(pmol/h)		(pmol/h)	
Membranes	94.9 $\pm$ 10.1	100	28.7 $\pm$ 5.2	100
100 000 g supernatant	75.5 $\pm$ 8.4	79	22.7 $\pm$ 1.3	79
Pellet	4.7 $\pm$ 1.1	5	1.22 $\pm$ 0.6	4.5
Detergent-poor phase	56.8 $\pm$ 7.4	60	18.4 $\pm$ 2.3	64
Detergent-rich phase	10.6 $\pm$ 0.9	11	2.6 $\pm$ 0.5	9

Figure 2 Kinetic parameters of membrane-associated PCMTs from the kidney cortex for AdoMet and ovalbumin

Solubilized BBM (○) or BLM (●) proteins (50 μ g) were incubated at 37 °C for 30 min in 100 mM Hepes/Tris (pH 7.5) with (a) 360 μ M of ovalbumin and increasing concentrations of [3 H]AdoMet (2 μ M), or (b) 2 μ M [3 H]AdoMet and increasing concentrations of ovalbumin. The kinetic parameters were estimated by non-linear regression analysis of the data, assuming Michaelis–Menten kinetics. The means $\pm$ S.D. of data obtained from four distinct experiments performed in triplicate are shown.

Figure 3 Identification of membrane-associated methyl-accepting proteins from BBM and BLM

Membrane proteins from BLM (lane 1) and BBM (lane 2) (100 μ g) were incubated at 37 °C for 60 min in 100 mM Hepes/Tris (pH 7.5) with 5 μ M [3 H]AdoMet (5 μ Ci). The methylation reaction was stopped by the addition of an equal volume of 2 $\times$ concentrated acidic sample buffer containing 10% (w/v) 16-BAC, and the samples were applied, without heating, on 7.5%-acrylamide gels. Electrophoresis was carried out overnight at a constant current of 30 mA, and the resulting gels were stained and destained as described in the Materials and methods section. The gels were soaked for 30 min in 1 M sodium salicylate, dried under low heat and exposed to Kodak XAR films at -70 °C for at least 1 month. A representative fluorogram is shown. Positions of standards of known molecular masses (kDa) are indicated (and in Figure 4).

subsequent phase partitioning, both enzymes were found to be predominantly associated with the more hydrophilic, detergent-poor, fraction (84 and 85% of the solubilized activities). These data thus suggest that, although both enzymes are tightly associated with the membranes, they are not intrinsically very hydrophobic molecules.

Properties of membrane-associated L-Isoaspartyl PCMTs

Experiments were performed to compare some structural and kinetic properties of the two membrane-associated enzymes. We first determined the apparent kinetic parameters of the two enzymes towards AdoMet, the methyl donor, and ovalbumin, a methyl acceptor. As shown in Figure 2, both enzymes presented very similar affinity towards these substrates, with similar K_m values, as determined by non-linear regression analysis, assuming Michaelis-Menten kinetics. The apparent K_m values (means $\pm$ S.E.M.) for AdoMet were 2.4 ± 0.3 and 2.7 ± 0.2 μ M for the BBM and BLM enzymes respectively, whereas K_m values for ovalbumin were of 25 ± 4 and 23 ± 3 μ M. These K_m values are similar to those reported for most PCMTs (Ota and Clarke, 1990). The higher activity observed for the BBM enzyme (Figure 1) thus depends on a higher apparent V_{max} , than that of the BLM-associated PCMT (338 ± 5 versus 254 ± 6 pmol/h per mg).

Figure 4 Molecular-mass determination of the membrane-associated PCMTs from BBM and BLM under denaturing and non-denaturing conditions

(a) Solubilized membrane proteins from BBM (○) or BLM (●) (1 ml; 8 mg of protein) were applied to a Superdex 75 column previously equilibrated with 20 mM Tris/HCl (pH 8.0) and 0.05% CHAPS. The protein were eluted at a flow rate of 0.5 ml/min, and 0.25 ml fractions were collected. PCMT activity was assayed by incubating 20 μ l samples in the presence of 2 μ M [3 H]AdoMet and 360 μ M of ovalbumin. (b) Solubilized proteins from BBM (○) or BLM (●) (25 μ g) were dissolved in Laemmli sample buffer and applied, without boiling, to 12.5% polyacrylamide gels. Electrophoresis was carried out for 90 min at a constant voltage of 100 V. The resulting gel was carefully sliced in 1 mm sections, which were then placed in 1.5 ml polypropylene tubes containing 200 μ l of 1% (w/v) Triton X-100, 50 mM Hepes/Tris (pH 7.5) and 15 mM β -mercaptoethanol. Proteins were eluted from the gel sections by overnight shaking of the tubes at room temperature. The L-Isoaspartyl PCMT activity was measured by incubating 20 μ l of the mixtures at 37 °C for 90 min in 100 mM Hepes/Tris (pH 7.5), 2 μ M [3 H]AdoMet and 360 μ M ovalbumin. Results are the means of five distinct experiments.

The endogenous substrates of the two membrane-bound enzymes were also compared, by using acidic gel electrophoresis in the presence of the cationic detergent 16-BAC. These conditions are essential to avoid hydrolysis of the methylated substrates (Barten and O'Dea, 1990). As shown in Figure 3, distinct membrane-associated methyl-accepting proteins were identified in both membranes. Proteins of 35, 50 and 54 kDa were found to be major substrates in the BBM, whereas proteins of 97 and 100 kDa were predominantly methylated in the BLM. The incorporation of radioactivity into these proteins was highly sensitive to the moderately alkaline conditions used in SDS/PAGE, suggesting that the proteins were methylated on L-Isoaspartyl residues (Clarke, 1985).

Gel filtration of solubilized BBM and BLM proteins revealed some differences in the elution profile of the membrane-associated PCMTs. As shown in Figure 4(a), the BLM-associated PCMT was eluted slightly earlier (1 ml) than the BBM enzyme during chromatography on a Superdex 75 column. The molecular masses of the enzymes obtained by gel filtration were 29 and 35 kDa for the BBM and BLM PCMTs respectively. To characterize further

Figure 5 Mono Q anion-exchange chromatography of BBM- and BLM-associated PCMTs

The pooled and concentrated active fractions from the Superdex 75 column (1 ml; 1 mg of protein) were applied to a Mono Q HR 5/5 column which had been previously equilibrated with 20 mM Tris/HCl and 0.05% CHAPS (pH 8.0). Proteins were eluted at a flow rate of 0.7 ml/min, with an exponential NaCl gradient, and 0.5 ml fractions were collected. Results are expressed as the percentage of activity of the most active fractions [BBM (○), 80 000 c.p.m.; BLM (●), 35 000 c.p.m.]. The results shown are representative of three experiments performed with different membrane preparations.

this difference in the sizes of the two enzymes, the electrophoretic mobilities of the membrane-associated enzymes were also compared, by using SDS/PAGE and subsequent renaturation of the enzymes. As shown in Figure 4(b), a significant difference in the electrophoretic mobility of the two enzymes could be observed after renaturation of sliced regions of the gel. The BBM-associated enzyme migrated in the gel with an apparent molecular mass of 27 kDa, whereas the BLM-associated PCMT activity was recovered in the area of the gel corresponding to a molecular mass of 29 kDa. These differences were highly reproducible, and were not affected by prior treatment of the solubilized enzymes by endoglycosidase F, a non-specific glycosidase for N-linked oligosaccharides (results not shown).

The fractionation of solubilized plasma membranes on the anion-exchanger Mono Q also revealed significant differences in the elution pattern of the membrane-associated enzymes (Figure 5). The BBM-associated PCMT activity was recovered in the flow-through of the column, and no detectable level of methylation activity could be released by the salt gradient. However, for the BLM-associated PCMT, we observed two distinct peaks of activity, a minor peak associated with the flow-through and a major peak eluted at 0.15 M NaCl (Figure 5). Similar profiles were obtained when the elution was performed with 0.5% CHAPS instead of 0.05%, suggesting that the differences were not related to non-specific protein aggregation (results not shown).

DISCUSSION

In the present work, we have studied the subcellular distribution of membrane-associated L-isopartyl PCMTs from rat kidney cortex. This tissue represents a useful model for such studies, since it can be fractionated by well-established techniques, and the isolated membranes possess very distinct protein compositions and enzymic properties (Simons and Fuller, 1985).

Prior treatment of the membranes with the zwitterionic detergent CHAPS strongly stimulated the carboxyl methylation of

L-isopartyl PCMT substrates, such as ovalbumin and the synthetic isopeptide TS-isoD-YSKY, by the purified plasma-membrane fractions. The effect of CHAPS was more pronounced for the BBM than for the BLM, with an 8-fold (compared to 2-fold) stimulation of the initial rate of methylation activity over non-detergent-treated membranes. This may be explained by the propensity of the BBM to form vesicles in iso-osmotic solutions, whereas BLM have a more sheet-like structure. This would result in a greater accessibility of the substrates for the BLM enzyme in the absence of detergent, and thus explain the greater activity in BLM in the absence of CHAPS.

The enzymes appear rather strongly associated with their respective membranes, since significant release of the enzymes was achieved only with relatively high amounts of detergent, but not with high-salt solutions. Interestingly, the Triton X-114 phase partitioning of the PCMTs into the detergent-poor fraction suggests that they are quite hydrophilic proteins, rather than integral or glycolipid-anchored membrane proteins (Hooper and Turner, 1988; Sargiacomo et al., 1989). However, the nature of this membrane interaction remains to be determined, since the concentration of Triton X-114 remaining in the aqueous phase (0.05%) is sufficient to solubilize some integral proteins.

We have recently reported the purification of the BBM-associated PCMT and showed that this enzyme appears very similar to the cytosolic PCMT in terms of its catalytic and structural properties (Boivin et al., 1993). In this paper, we show that the membrane-associated PCMTs from BBM and BLM also appear homologous in their catalytic properties, as they methylate an exogenous protein, such as ovalbumin, and a synthetic L-isopartyl-containing peptide, and possess similar apparent affinities for both methyl donor and acceptor. However, the two enzymes were found to be distinct in other aspects. The BLM-associated enzyme has a slightly higher molecular mass than the BBM PCMT under both denaturing and non-denaturing conditions. An apparent molecular mass of 29 kDa was estimated for the BLM PCMT by SDS/PAGE, compared with 27 kDa for the BBM PCMT. This difference did not appear to be related to a differential glycosylation pattern of the two enzymes, since treatment of the proteins with endoglycosidase F before the electrophoresis had no effect on their mobilities. It is noteworthy that, although the molecular mass of L-isopartyl PCMTs is generally close to 27 kDa (Clarke, 1985), previous reports have documented the existence of distinct forms of PCMT with different molecular masses. In *Torpedo ocellanata*, two distinct PCMTs of 27 and 35 kDa have been described (Solomon et al., 1988), and two forms of PCMT, with distinct subcellular localization, have been identified in *Xenopus laevis* oocytes (27 and 34 kDa) (O'Connor, 1987).

Chromatographic separation of solubilized plasma-membrane proteins according to ionic charge also showed the presence of distinct forms of PCMT in each membrane. BBM contains exclusively a basic PCMT isoenzyme, which did not bind to the Mono Q column, whereas BLM contains predominantly a more acidic PCMT isoenzyme that was eluted with a salt gradient. Such a separation of two forms of PCMT by anionic exchangers have been frequently observed for the cytosolic enzymes from various tissues (Cusan et al., 1981; Aswad and Deight, 1983; Ota et al., 1988). In those cases, the isoenzymes have identical kinetic properties and highly homologous structures (Ota et al., 1988; Potter et al., 1992; MacLaren et al., 1992). For the brain and erythrocyte isoenzymes, the only difference was in the C-terminal region of the proteins, where the C-terminal sequence of the more acidic forms of the enzymes ends in -RDEL, compared with -RWK for the more basic isoenzymes (Potter et al., 1992; MacLaren et al., 1992). These substitutions seem to arise from

the alternative splicing of the last exon of the gene encoding the PCMT, resulting in the formation of distinct mRNAs (Romanik et al., 1992; MacLaren et al., 1992). Although it is not clear at present whether the distinct PCMTs identified in the kidney cortex are derived from a single gene which has undergone alternative splicing, it is noteworthy that three distinct mRNAs for the PCMT have been detected in mouse kidney (Romanik et al., 1992).

The molecular basis and physiological significance of the asymmetrical distribution of L-isoaspartyl PCMTs in two distinct plasma membrane domains of the kidney cortex remains to be clarified. In these cells, the targeting of membrane proteins is a complex process which involves the recognition of specific signals imbedded in the protein structure (Mostov et al., 1992), and adequate targeting is critical for the maintenance of cell polarity and function (Rodriguez-Boulán and Nelson, 1989). Thus the differential targeting of L-isoaspartyl PCMTs may reflect an important function played by these enzymes, which could involve the recognition of membrane-associated proteins specific for each type of membranes.

The physiological functions of membrane-associated L-isoaspartyl PCMTs are yet to be defined. After their extraction from the membranes with a detergent, these enzymes appear very similar to the cytosolic enzymes with regard to their catalytic and structural properties (Brown, 1984; Iqbal and Steenson, 1976; Boivin et al., 1993), suggesting a similar physiological role. In this context, it is noteworthy that increased deamidation of membrane-associated substrates by alkaline treatment results in an increased activity of the brain membrane-associated methyltransferase (Sellinger et al., 1987). The role of the membrane-associated PCMTs may thus be similar to that of the cytosolic one, and could involve recognition of L-isoaspartyl-containing proteins not accessible to the cytosolic enzyme *in vivo*.

We thank Lise Tremblay for helpful discussions and Dr. Vincent Vachon for his critical reading of the manuscript. The financial support of the Kidney Foundation of Canada is gratefully acknowledged.

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