Mammalian protein methylesterase

Physical and enzymic properties

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Protein methylesterase (PME) amino acid composition and substrate specificity towards methylated normal and deamidated protein substrates were investigated. The enzyme contained 23 % acidic and 5 % basic residues. These values are consistent with a pI of 4.45. The product formed from methylated protein by PME was confirmed as methanol by h.p.l.c. The k_{cat} and K_m values for several methylated protein substrates ranged from 20×10^{-6} to $560 \times 10^{-6} \text{ s}^{-1}$ and from 0.5 to $64 \mu\text{M}$ respectively. However, the k_{cat}/K_m ratios ranged within one order of magnitude from 11 to $52 \text{ M}^{-1} \cdot \text{s}^{-1}$. Results with the irreversible cysteine-proteinase inhibitor E-64 suggested that these low values were in part due to the fact that only one out of 25 molecules in the PME preparations was enzymically active. When PME was incubated with methylated normal and deamidated calmodulin, the enzyme hydrolysed the latter substrate at a higher rate. The K_m and k_{cat} for methylated normal calmodulin were $0.9 \mu\text{M}$ and $31 \times 10^{-6} \text{ s}^{-1}$, whereas for methylated deamidated calmodulin values of $1.6 \mu\text{M}$ and $188 \times 10^{-6} \text{ s}^{-1}$ were obtained. The k_{cat}/K_m ratios for methylated normal and deamidated calmodulin were 34 and $118 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. By contrast, results with methylated adrenocorticotrophic hormone (ACTH) substrates indicated that the main difference between native and deamidated substrates resides in the K_m rather than the k_{cat} . The K_m for methylated deamidated ACTH was 5-fold lower than that for methylated native ACTH. The k_{cat}/K_m ratios for methylated normal and deamidated ACTH were 43 and $185 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively. These results indicate that PME recognizes native and deamidated methylated substrates as two different entities. This suggests that the methyl groups on native calmodulin and ACTH substrates may not be on the same amino acid residues as those on deamidated calmodulin and ACTH substrates.

INTRODUCTION

The ubiquitous enzyme protein-carboxyl methylase (PCM; *S*-adenosylmethionine:protein-L-glutamate *O*-methyltransferase, EC 2.1.1.24) esterifies free carboxy groups of proteins, resulting in the neutralization of their negative charges (Liss *et al.*, 1969; Kim & Paik, 1970, 1971; Morin & Liss, 1973). Several laboratories have provided evidence implicating eukaryotic PCM as a regulatory agent in a wide variety of cellular processes, including storage and release of secretory proteins (Diliberto *et al.*, 1976; Borchardt *et al.*, 1978; Gagnon *et al.*, 1978; Heisler *et al.*, 1983; Kloog & Saavedra, 1983), modulation of acetylcholine receptors (Kloog *et al.*, 1980; Flynn *et al.*, 1982; Nuske, 1986) and dopamine receptors (Billingsley & Roth, 1982), regulation of calmodulin activity (Gagnon *et al.*, 1981; Billingsley *et al.*, 1983), sperm motility (Bouchard *et al.*, 1980), chemotaxis of leucocytes (O'Dea *et al.*, 1978b; Pike *et al.*, 1978) and slime moulds (Mato & Marin-Cao, 1979), platelet function (O'Dea *et al.*, 1978a), development of membrane excitability in neurons (Kloog *et al.*, 1983) and sensory transduction in the vertebrate rod outer

segment (Swanson & Applebury, 1983). However, by contrast with PCM involvement in bacterial chemotaxis, strong and direct evidence for a specific regulatory function of protein carboxy-group methylation in eukaryotic cells is still lacking (Clarke & O'Connor, 1983; Clarke, 1985).

By contrast with the numerous reports on PCM, little is known about protein methylesterase (PME), an enzyme that hydrolyses protein methyl esters formed by PCM into methanol and unmethylated proteins. In mammalian cells PME was originally thought not to exist, since protein methyl esters were spontaneously hydrolysed under physiological conditions (Kim & Paik, 1976; Gagnon *et al.*, 1978). However, PME was detected in all tissues investigated. Its level of activity varied more than 100-fold from one tissue to the other, the richest source being kidneys (Chene *et al.*, 1982). Purified kidney PME appears to be made of a single polypeptide chain with an M_r of 31 000 (Chene *et al.*, 1982). By contrast, the bacterial PME has an M_r of 37 000 (Snyder *et al.*, 1984). Oligomers of M_r 100 000 have also been reported for the bacterial PME (Stock & Koshland, 1978).

On a functional point of view, bacterial PME plays an

Abbreviations used: Ado[³H]Met, *S*-adenosyl[³H]methionine; E-64, *N*-[*N*-(1-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine; ACTH, adrenocorticotrophic hormone (corticotropin); PCM, protein-carboxyl methylase; PME, protein methylesterase.

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important role in chemotaxis (Stock & Koshland, 1978; Springer *et al.*, 1979; Toews & Adler, 1979). Mammalian PME has been shown to be stimulated within minutes when rabbit neutrophils were incubated with the synthetic chemoattractant fMet-Leu-Phe through a receptor-mediated mechanism (Venkatasubramanian *et al.*, 1980). A 7–20-fold decrease in PME activity has also been reported in spermatozoa maturing through the epididymis (Gagnon *et al.*, 1984b).

In the present paper we report some of the properties of PME with regard to its amino acid composition, active site and substrate specificity towards various native and deamidated methylated proteins.

MATERIALS AND METHODS

Materials

S-Adenosyl[Me-³H]methionine (Ado[³H]Met) (15–28 Ci/mmol) was obtained from Amersham Corp. (Toronto, Ont., Canada) and ICN Radiochemicals (Montreal, Que., Canada); S-adenosyl-L-methionine and E-64 {N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine} was from Boehringer-Mannheim (Montreal, Que., Canada); Sephadex G-25 and calmodulin were from Pharmacia Fine Chemicals (Montreal, Que., Canada); ovalbumin, gelatin (pig skin), bovine γ -globulin and histone (calf thymus) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.); sheep growth hormone (somatotropin), follicle-stimulating hormone (follictropin), luteinizing hormone (lutropin) and rat prolactin were from the National Hormone and Pituitary Program (Baltimore, MD, U.S.A.); synthetic human adrenocorticotrophic hormone (ACTH, corticotropin) was from CIBA-GEIGY (Basel, Switzerland). Seminal plasma was obtained from human semen following a 20000 g centrifugation at 4 °C.

Purification of PCM

PCM was purified from the cytosol of bovine erythrocytes as previously described (Gagnon, 1979). The purification steps involved: (1) a 55% satd.-(NH₄)₂SO₄ precipitation of erythrocyte cytosol, (2) chromatography on Sephadex G-100, (3) a second 55% satd.-(NH₄)₂SO₄ precipitation and (4) chromatography on hydroxyapatite. The purified preparation was devoid of endogenous methyl-acceptor proteins. The enzyme preparation used in these experiments had a specific activity of 9636 pmol/min per mg of protein at 37 °C with ovalbumin (dialysed against water) as the methyl acceptor.

Purification of PME

PME was purified from rat kidneys as previously described (Gagnon *et al.*, 1984a). The purification steps involved (1) 65% satd.-(NH₄)₂SO₄ precipitation of kidney cytosol, (2) chromatography on Sephadex G-100 after dialysis at pH 4.35, (3) chromatography on Polybuffer exchanger 94 and (4) chromatography on Matrex Gel Green A. Dithiothreitol (0.1 mM) was present in all buffers used in the purification procedure.

PME reaction

PME activity was measured by the method of Gagnon *et al.* (1984a). Two enzymic reactions are involved in this assay. For the first reaction (methylation of proteins), substrates (0.25–7.35 μ M) were incubated with 1.73–

88 μ M-Ado[³H]Met (specific radioactivities from 0.88 to 28 Ci/mmol) and 0.2–2.6 μ M-PCM in 62.5 mM-sodium acetate buffer, pH 6.0. After a 30 min incubation at 37 °C the reaction was stopped by the addition of 2 mM-S-adenosylhomocysteine and 0.2 M-sodium acetate buffer, pH 4.35. Radioactive protein methyl esters were then separated from unused Ado[³H]Met by chromatography on Sephadex G-25 that had been equilibrated in 0.25 M-sodium acetate buffer, pH 4.35, containing 0.1 mM-dithiothreitol and 5% (v/v) glycerol. For the second reaction, the isolated protein [³H]methyl esters were then incubated with a PME preparation at 37 °C in a final volume of 70 μ l. This reaction was terminated by the addition of 15% (w/v) trichloroacetic acid, and the [³H]methanol formed was extracted and its radioactivity counted as previously described (Gagnon, 1979).

Identification of the PME product by h.p.l.c.

Radioactive methanol as the product of PME enzymic activity on protein [³H]methyl esters was quantitatively determined by the SP 8100 XR extended-range h.p.l.c. system from Spectra Physics (Technical Marketing Associates, Montreal, Que., Canada). Methanol was separated on a Polypore H (4.6 mm $\times$ 220 mm) column equilibrated in 5 mM-H₂SO₄. Methanol elution was monitored by its refractive index measured by a SP 6040 XR RI detector from Spectra Physics. Ovalbumin [³H]methyl esters (677629 c.p.m.; 22 pmol) in a total volume of 70 μ l were incubated with 31–245 pmol of PME at 37 °C for 20 min. After 20 min 120 μ l of 5 mM-H₂SO₄ and 10 μ l of non-radioactive methanol (0.5 g/l) were added to the reaction mixture. Samples were frozen in solid CO₂ until analysed by h.p.l.c. the same day. The column was equilibrated in 5 mM-H₂SO₄ and the column temperature was maintained at 40 °C. Under a flow rate of 0.5 ml/min and a 4.1–4.5 MPa (600–650 lbf/in²) pressure, methanol was eluted at 6.2 min. Radioactivity in the eluted fraction was measured by liquid-scintillation counting. The amount of radioactivity in the methanol peak was compared with that in parallel incubation mixtures where the methanol produced during incubation was evaluated by the toluene/isopentanol (3:2, v/v) procedure as described previously (Gagnon, 1979).

Amino acid analysis

Purified PME preparations were subjected to SDS/polyacrylamide-gel electrophoresis, and the major 31000-M_r band was cut out and extracted as described elsewhere (Hanaoka *et al.*, 1979; Higgins & Dahmus, 1979). About 2–3 mg of electroeluted protein was extensively dialysed against 1 mM-potassium phosphate buffer, pH 7.0, taken in cleaned acid-hydrolysis tubes and dried by speed vacuum centrifugation. Acid-hydrolysis operations were performed with a Pico-Tag Work Station (Waters, Division of Millipore) by using the following methods. HCl (Pierce Chemical Co.) containing phenol (1%, v/v) was added to the bottom of the hydrolysis container, which was purged three times with N₂ as prescribed by Waters. Then the container was sealed under reduced pressure and placed in the heating block set at 110 °C for 24, 48 and 72 h. Analyses were performed on a Beckman System 6300 High Performance Analyser according to the general procedures of Spackman *et al.* (1958). Half-cystine content was determined by measuring the change in absorbance at

412 nm during reaction of NaBH_4 -reduced and SDS-denatured enzyme with 5,5'-dithiobis-(2-nitrobenzoic acid) (Habeeb, 1972). Tryptophan was determined spectrophotometrically (Edelhoch, 1967). Methionine residues were not oxidized before or during HCl hydrolysis.

Deamidation reaction

A 2 mg portion of synthetic human ACTH and 3.5 mg of calmodulin were dissolved in a final volume of 0.5 ml containing 0.1 M- NH_3 , pH 11.0, and incubated at 37 °C for 3 h. The sample was then adjusted to pH 6 by addition of 1.0 M-acetic acid and freeze-dried.

Kinetic data

The initial hydrolysis rates of the $[^3\text{H}]$ methylated proteins by PME were determined by measuring the $[^3\text{H}]$ methanol liberated. The kinetic constants K_m and V_{max} were derived from linear-regression analysis and were based on quadruplicate determinations of the initial rates at a minimum of eight substrate concentrations.

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

RESULTS

Amino acid composition

The amino acid composition of purified PME was deduced from acid hydrolysates at 24, 48 and 72 h. The acidic amino acids aspartic acid and glutamic acid represented 23% of the total amino acid residues, whereas the basic amino acids lysine, histidine and

arginine contributed only 5% of PME residues. Glycine, the predominant neutral amino acid, accounted for up to 17% of the total amino acid residues (Table 1).

Methanol as the product of PME hydrolysis

Ovalbumin $[^3\text{H}]$ methyl esters (677 629 c.p.m.; 22 pmol) were incubated with 122.5 pmol of PME, and the $[^3\text{H}]$ methanol liberated was extracted with the standard toluene/isopentanol (3:2, v/v) procedure (Gagnon, 1979). Parallel reaction mixtures were also analysed by h.p.l.c. to confirm the hydrolytic products. Following h.p.l.c., the radioactivity was completely recovered under the methanol peak, indicating that the product formed by PME action and measured by the standard toluene/isopentanol extraction procedure was $[^3\text{H}]$ methanol only (Fig. 1). The quantities of $[^3\text{H}]$ methanol recovered in the methanol peak represented 96–106% of the radioactivity recovered as methanol by the toluene/isopentanol procedure (Gagnon, 1979) (Table 2). Methanol, the first alcohol eluted with this h.p.l.c. system, had a retention time of 6.19–6.22 min. H.p.l.c. analyses were performed beyond 10 min, but no other peak was observed.

Substrate specificity

PME substrate specificity was investigated by incubating methyl esters of various proteins [ovalbumin, growth hormone, luteinizing hormone, seminal-plasma proteins, histone, bovine γ -globulin, gelatin, follicle-stimulating hormone, prolactin, calmodulin (native) and ACTH (native)] with PME. The K_m values for the various substrates varied from 0.5 μM for histone methyl ester to 63 μM for bovine γ -globulin methyl ester (Table 3). There was a 28-fold difference in the k_{cat} values for histone methyl ester ($20 \times 10^{-6} \text{ s}^{-1}$) and for prolactin methyl ester ($560 \times 10^{-6} \text{ s}^{-1}$). However, the k_{cat}/K_m ratios ranged within one order of magnitude from 11 to 52 $\text{M}^{-1} \cdot \text{s}^{-1}$.

Table 1. Amino acid composition of PME from rat kidneys

Quadruplicate samples were analysed after 24 h, 48 h and 72 h hydrolysis in 6 M-HCl. Threonine and serine were determined by extrapolation to zero time of hydrolysis. Half-cystine was determined by the method of Habeeb (1972). Tryptophan was determined by the method of Edelhoch (1967).

Amino acid	Composition (mol/100 mol of residues)
Asx	7.8
Thr	6.8
Ser	17.7
Glx	15.2
Gly	16.6
Ala	6.3
Cys	2.1
Val	3.9
Met	0.9
Ile	2.9
Leu	4.4
Tyr	2.4
Phe	2.3
His	1.9
Lys	1.6
Trp	2.6
Arg	1.9
Pro	2.7

Fig. 1. H.p.l.c. analyses of $[^3\text{H}]$ methanol produced by the PME reaction

Ovalbumin $[^3\text{H}]$ methyl esters (677 629 c.p.m.; 22 pmol) were incubated with 122.5 pmol of PME for 20 min at 37 °C in a total volume of 70 μl . The reaction was arrested and the methanol liberated was determined ($\bigcirc$ — $\bigcirc$) by h.p.l.c. as described in the Materials and methods section. —, Relative refractive index.

Table 2. Product analysis of ovalbumin [³H]methyl esters incubated with PME

Ovalbumin [³H]methyl esters (677629 c.p.m.) were incubated with PME or 1 M-NaOH (5 min). For details see the Materials and methods section. [³H]Methanol formed was extracted with toluene/isopentanol (3:2, v/v) with an extraction efficiency of 85 % and measured as the difference between evaporated and non-evaporated portions of the organic phase (Gagnon, 1979). [³H]Methanol formed was also separated by h.p.l.c. after the addition of unlabelled methanol. The radioactivity in the methanol peak at 6.2 min was counted in a liquid-scintillation spectrometer.

Treatment	[³ H]Methanol formed (c.p.m.)		100 × (B/A ratio) (%)
	Organic extraction (A)	H.p.l.c. method (B)	
1. 1 M-NaOH	677629	628866	93
2. PME			
0.44 μM	16835	15792	96
0.88 μM	28926	30772	106
1.75 μM	64352	62544	97
2.6 μM	119657	120528	101
3.5 μM	143305	144558	101

Table 3. Kinetic parameters for the hydrolysis of [³H]methylated proteins by PME

Methylated proteins were incubated with 122.5 pmol of PME for 10 min at 37 °C in a total volume of 70 μl and the radioactive methanol produced was determined as described elsewhere (Gagnon, 1979). Values are means ± S.D. for at least four determinations.

Substrate	K_m (μM)	$10^6 \times k_{cat}$ (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)
Ovalbumin	7.2 ± 2.6	356 ± 77	52 ± 8
Growth hormone	17.4 ± 7.0	432 ± 124	33 ± 10
Luteinizing hormone	1.9 ± 0.6	74 ± 28	45 ± 4
Seminal-plasma proteins	2.1 ± 0.7	99 ± 12	49 ± 9
Histone	0.5 ± 0.1	20 ± 6	50 ± 7
Bovine γ-globulin	63 ± 31	462 ± 244	11 ± 4
Gelatin	1.3 ± 0.5	54 ± 17	47 ± 14
Follicle-stimulating hormone	2.4 ± 2	169 ± 27	49 ± 24
Prolactin	26 ± 4	560 ± 74	22 ± 1
Calmodulin (native)	0.9 ± 0.2	31 ± 3	34 ± 5
ACTH (native)	2.6 ± 0.4	110 ± 13	43 ± 2

Inhibition of PME activity by E-64

We have previously reported the inhibition of PME activity by leupeptin and chymostatin (Veeraragavan & Gagnon, 1987). We observed that one molecule of either of these inhibitors blocked the activity of several enzyme molecules. We also observed that cysteine was one of the amino acid residues present in the active site (K. Veeraragavan & C. Gagnon, unpublished work). To determine the exact number of inactive molecules in the PME preparation, a sample was treated with E-64, a cysteine-proteinase inhibitor that inactivates enzymes by forming an irreversible thioether bond (Hanada *et al.*,

Fig. 2. Inhibition of PME activity by various concentrations of E-64

Three amounts of PME [122.5 pmol (●), 184 pmol (▲) and 245 pmol (■)] were incubated with various amounts of E-64 at 37 °C for 5 min in a total volume of 30 μl. Following this preincubation, 40 μl of ovalbumin [³H]methyl esters (244239 c.p.m.; 252 pmol) were added and the incubation was continued for a further 20 min. The reaction was then arrested and the [³H]methanol liberated was determined.

1978a,b). It was found that a 5 min preincubation was sufficient to block the PME activity completely. Hence, for incubation studies, various concentrations of PME were incubated with E-64 for 5 min and then substrate was added to the incubation mixture to measure the remaining enzyme activity. From inhibition curves at three enzyme concentrations it was calculated that 1 molecule of E-64 inhibited the activity generated from 23–25 molecules of PME (Fig. 2).

PME hydrolysis of methylated native and deamidated calmodulin

Previous experimental evidence suggests that deamidated proteins are excellent substrates for PCM (Aswad, 1984; Johnson *et al.*, 1985). We confirmed that deamidated calmodulin always proved to be a better substrate for PCM than native calmodulin. Our results indicate extents of methylation for native and deamidated calmodulin of 0.03 mol and 0.49 mol/mol of calmodulin respectively. When PME was incubated with identical amounts of methyl esters of these two substrates, PME hydrolysed methyl esters of deamidated calmodulin at a higher rate than those of native calmodulin. Under initial-velocity conditions, an apparent K_m of 0.9 μM and a k_{cat} of $31 \times 10^{-6} \text{ s}^{-1}$ was observed for methylated native calmodulin (Table 4). The apparent K_m of 1.6 μM for the methylated deamidated calmodulin was similar to that of methylated native calmodulin, but its k_{cat} was 6-fold higher. The k_{cat}/K_m ratio for methylated deamidated calmodulin was 3.5-fold higher than that for methylated native calmodulin (Table 4). These results suggest that methylated native calmodulin and methylated deamidated calmodulin were considered by PME as two different substrates.

PME hydrolysis of methylated native and deamidated human ACTH

As for calmodulin, we confirmed the results reported by Aswad (1984) showing that deamidated ACTH was a far better substrate for PCM than was native ACTH. Deamidated ACTH incorporated up to 0.68 mol of methyl group/mol of hormone whereas native ACTH

Table 4. Kinetic parameters for the hydrolysis of [³H]methylated native and deamidated calmodulin and ACTH by PME

Methylated substrates were incubated with 122.5 pmol of PME for 10 min at 37 °C in a total volume of 70 μ l and the radioactive methanol was determined as described elsewhere (Gagnon, 1979). Values are means $\pm$ S.D. for at least four determinations.

Substrate	K_m (μ M)	$10^6 \times k_{cat}$ (s ⁻¹)	k_{cat}/K_m (M ⁻¹ ·s ⁻¹)
Calmodulin			
Native	0.9 $\pm$ 0.2	31 $\pm$ 3	34 $\pm$ 5
Deamidated	1.6 $\pm$ 0.1	188 $\pm$ 12	118 $\pm$ 2
ACTH			
Native	2.6 $\pm$ 0.4	110 $\pm$ 13	43 $\pm$ 2
Deamidated	0.5 $\pm$ 0.1	95 $\pm$ 2	185 $\pm$ 35

Table 5. Effect of deamidated calmodulin and ACTH on PME rate of hydrolysis of methylated deamidated calmodulin and ACTH

Conditions	Molar ratio*	PME activity (% of control)
Calmodulin		
Control	—	100
Deamidated calmodulin†	2	97
	9	102
	39	94
ACTH		
Control	—	100
Deamidated ACTH‡	2	104
	9	110
	39	104

* Ratio between deamidated calmodulin or deamidated ACTH and methylated deamidated substrate.

† Deamidated calmodulin (0.032, 0.144 and 0.624 nmol) was incubated with 0.18 nmol of PME for 20 min at 37 °C. The [³H]methanol formed was determined as described in the Materials and methods section.

‡ Deamidated ACTH (0.02, 0.09 and 0.39 nmol) was incubated with 0.25 nmol of PME for 20 min at 37 °C. The [³H]methanol formed was determined as described in the Materials and methods section.

incorporated only 0.08 mol of methyl group/mol of hormone. As with the methyl esters of native and deamidated calmodulin, PME hydrolyses methyl esters of deamidated ACTH at a faster rate than those of native ACTH. Under initial-velocity conditions an apparent K_m of 2.6 μ M and a k_{cat} of 110×10^{-6} s⁻¹ were observed for methylated native ACTH, whereas the corresponding values were 0.5 μ M and 95×10^{-6} s⁻¹ respectively for methylated deamidated ACTH (Table 4). The k_{cat}/K_m ratio for methylated deamidated ACTH was 4.3-fold higher than that for methylated native ACTH. These results suggest that methylated native ACTH and methylated deamidated ACTH were considered as two unrelated substrates with respect to PME.

Effect of deamidated calmodulin and ACTH on PME rate of hydrolysis of methylated deamidated ACTH and calmodulin

Since for the kinetic studies described above with methylated deamidated calmodulin and ACTH the methylated substrates were simply diluted to the appropriate concentrations, we investigated whether the presence of non-methylated deamidated peptides could influence PME activity. The presence of up to a 39-fold excess in non-methylated deamidated peptides did not influence the rate of hydrolysis of methylated deamidated calmodulin and ACTH (Table 5).

DISCUSSION

We have previously purified PME from rat kidneys and shown that this enzyme had an isoelectric point of 4.45 (Gagnon *et al.*, 1984a). The amino acid analysis reported in Table 1 showing the presence of 23 % acidic and 5 % basic residues is consistent with the low isoelectric point reported for mammalian PME.

We have also previously shown that leupeptin and chymostatin are two powerful inhibitors of PME activity as measured by its capacity to hydrolyse protein methyl esters into a volatile product extractable by toluene/isopentanol (3:2, v/v) (Veeraragavan & Gagnon, 1987). H.p.l.c. analysis of this product confirmed that the hydrolytic product of ovalbumin [³H]methyl esters was [³H]methanol only. These results clearly indicate that even if PME is inhibited by the proteinase inhibitors mentioned above, it generates only a trichloroacetic acid-soluble and organic-solvent-extractable product in the form of methanol from protein methyl ester substrates.

It has been reported that deamidated calmodulin and ACTH were far better substrates for PCM than were the native proteins (Aswad, 1984; Johnson *et al.*, 1985). We have confirmed these results. Thus spontaneous deamidation of calmodulin may explain the high extent of methylation (up to 0.5 mol/mol of calmodulin) previously reported (Gagnon *et al.*, 1981; Johnson *et al.*, 1985). On the other hand, carboxy-group methylation of calmodulin in intact cells was clearly established (Gagnon *et al.*, 1981). The question that arises is whether the same carboxy group is methylated in native and deamidated proteins. Results with both ACTH and calmodulin suggest that these sites may be different, since the K_m and/or V_{max} values of PME for methylated native and deamidated peptides are different. However, the possibility that the differences in the kinetic parameters between methylated native and methylated deamidated peptides may be due to conformational changes due to deamidation cannot be discarded. According to Aswad (1984), deamidation occurs predominantly at Asn-Gly residues of the ACTH molecule, resulting in an isoaspartic acid residue that is being stoichiometrically methylated. Asparagine-25 of ACTH was shown to be methylated by PCM once it had been deamidated by alkaline treatment. However, whether the same amino acid residue was methylated in native ACTH was not established. The glutamic acid residue in position 28 previously reported by Kim & Li (1979) as a methylation site could be a potential candidate.

Not all proteins increase their methyl-accepting capacity once deamidated, suggesting that sites other than Asn-Gly can be used by PCM. For example, the

peptide hormones luteinizing hormone and follicle-stimulating hormone have three Asn-Gly pairs but the standard alkaline treatment that caused deamidation of ACTH and calmodulin did not increase the rate of methylation of these peptide hormones (K. Veeraragavan & C. Gagnon, unpublished work).

PME kinetic parameters for a variety of substrates were surprising. Whereas the majority of the K_m values were less than $5 \mu\text{M}$, with the lowest value at $0.5 \mu\text{M}$, the k_{cat} was very very low, with the maximal value reaching only $560 \times 10^{-6} \text{ s}^{-1}$. The k_{cat}/K_m ratio for the various substrates varies from 11 to $52 \text{ M}^{-1} \cdot \text{s}^{-1}$. Even though these kinetic parameters are unusual, PME is nevertheless an enzyme. It is thermolabile and degraded by trypsin (Gagnon, 1979), and it increases the rate of hydrolysis of protein methyl esters to give methanol. At pH 4.35 the spontaneous rate of hydrolysis of protein methyl esters under our experimental conditions is very low, and 1 nmol of PME will increase that rate 13000-fold.

When compared with the bacterial PME, mammalian PME appears to have a lower K_m and a lower V_{max} . Snyder *et al.* (1984) reported a K_m of $15 \mu\text{M}$ for the methyl-accepting chemotaxis proteins and a turnover number of 0.004 mol of methanol/s per mol of PME under saturation kinetic conditions. The bacterial k_{cat}/K_m ratio can be calculated to be $270 \text{ M}^{-1} \cdot \text{s}^{-1}$, which is within the same order of magnitude as the $52 \text{ M}^{-1} \cdot \text{s}^{-1}$ value of PME for ovalbumin methyl esters or $185 \text{ M}^{-1} \cdot \text{s}^{-1}$ for methylated deamidated ACTH. Thus bacterial PME and mammalian PME have similar catalytic properties with regard to their respective macromolecular substrates. Nevertheless, these turnover rates, which are similar to those for catalytic properties of antibodies (Hansen, 1987), are still very low when compared with most other enzymes. This may suggest (1) that PME is partially inactivated during the isolation and/or purification procedures, (2) that only some of the enzyme molecules are in an active form, (3) that our assay conditions *in vitro* are not optimal for PME activity, (4) that the hydrolysis of protein methyl esters to give methanol is a secondary reaction to a different enzymic activity and (5) that the hydrolysis of protein methyl esters to give methanol is the reverse reaction of a synthetic reaction.

The fact that PME was purified from kidney homogenate with a yield of 28% (Gagnon *et al.*, 1984a) rules out the first possibility, since after a five-step purification procedure a 28% recovery is normal. On the other hand, if inactivation occurred before or during homogenization, we would not have detected it. Our previous results with leupeptin and chymostatin suggested that less than 10% of the proteins present in the PME preparation are PME molecules (Veeraragavan & Gagnon, 1987). The present data with E-64 confirmed these results and suggested that not more than one molecule out of 25 was active. The presence of inactive molecules is often observed in thiol enzymes. For example, papain, a cysteine proteinase, has more molecules in inactive than in active forms. This phenomenon is caused by auto-oxidation and/or irreversible inactivation by several factors (Sluyterman, 1967; Sluyterman & Wijdenes, 1970). In addition, it has been demonstrated that the bacterial PME enzyme could be activated 15-fold as a result of partial digestion by trypsin (Borczuk *et al.*, 1986). The possibility that PME low turnover rates are partially due to the presence of

latent enzyme is a real one in both prokaryotic and eukaryotic cells. *In vivo*, cells may increase PME activity through transient activation, as is often achieved with a variety of other enzymes. *In vitro*, an enzyme is measured under specific conditions that may or may not be optimal for activity. When incubated with non-methylated proteins and methanol or AdoMet, PME was unable to form protein methyl esters, suggesting that PME does not catalyse a reverse reaction (results not shown). On the other hand, the possibility that PME catalyses other types of reactions is a real one. Preliminary results suggest that PME can also hydrolyse peptide bonds on small synthetic peptide substrates in addition to methyl esters (K. Veeraragavan & C. Gagnon, unpublished work). It is well known that some esterases are capable of hydrolysing peptide bonds (Walker & Mackness, 1983).

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