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PRESENCE OF A PROTEIN METHYLESTERASE IN MAMMALIAN TISSUES

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SUMMARY. Using enzymatically methylated protein-^{3}H methyl esters as substrates, a protein methylesterase activity was observed in mammalian tissues. The product of hydrolysis was identified as radioactive methanol by microdistillation under reduced pressure and formation of a 3,5-dinitrobenzoate derivative. The enzyme was active over a broad range of pH and appeared in all tissues investigated. Since protein carboxyl-methylation is involved in chemotaxis of leukocytes and exocytotic secretion, this protein methylesterase probably participates in the regulation of these processes.

Protein carboxyl-methylase (S-adenosyl-L-methionine: protein-carboxyl-O-methyltransferase, EC 2.1.1.24) methylates or esterifies free carboxyl groups of proteins and neutralizes negative charges on protein substrates (1-4). Although the majority of proteins have aspartic or glutamic acid residues in their primary structure, some proteins are much better substrates than others. Anterior pituitary peptide hormones (LH, GH, FSH, ACTH, etc ...), neurophysins and chromaffin granule soluble and membrane bound proteins are excellent substrates (5-9).

Protein carboxyl-methylation has been associated with secretion (6-8, 10-12) and with chemotaxis both in bacteria (13-16) and in leukocytes (17-18). Protein-methyl esters from mammals in comparison with those from bacteria are very unstable at physiological temperature and pH and their hydrolysis is thought to be spontaneous or non-enzymatic (4, 7, 19).

In this paper, I present evidence for the existence of a mammalian protein methylesterase. The enzyme is found in several tissues, with the highest concentration occurring in kidney.

MATERIAL AND METHODS

Materials. S-adenosyl-L-[methyl-^{3}H] methionine, 9.2 Ci/mmol, was purchased from New England Nuclear (Boston, Mass.). Pig skin gelatin, bovine

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pancreatic trypsin and soya bean trypsin inhibitor were obtained from Sigma Chemical (Saint-Louis, Mo); sucrose (ultrapure grade) from Schwarz/Mann (Orangeburg, N.J.); S-adenosyl-homocysteine from Boehringer Mannheim Biochemicals (Indianapolis, In.); 3,5-dinitrobenzoyl chloride from Eastman Kodak Co. (Rochester, N.Y.) and Nonidet P-40 from Bethesda Research Laboratories (Bethesda, M.D.).

Preparation of homogenates. Male Sprague-Dawley rats weighing 200-300 g were killed by decapitation. Various organs were collected and homogenized with 9 volumes of ice cold 0.3 M sucrose.

Protein methylesterase activity determination. Protein methylesterase activity was measured using enzymatically methylated protein-^{3}H methyl esters as substrates. The incubation mixture contained: 10 µl of 0.25 M Na acetate buffer pH 5.2, 10 µl of gelatin (50 mg/ml), 10 µl of purified protein carboxyl-methylase and 10 µl of S-adenosyl-^{3}H methyl-methionine 3.5 µM (1.25 µCi/10 µl). After a 10 min incubation, the reaction was stopped by the addition of 10 µl of 10 mM S-adenosyl-homocysteine. Boiled or fresh homogenates (20 µl) were then added and incubation proceeded for 20 more min at 37°C. At the end of the second incubation, 0.5 ml of 10% trichloroacetic acid was added and the precipitated proteins centrifuged at 20,000 x g for 10 min. The pelleted proteins were resuspended in 200 µl of 1M Na borate buffer pH 11.0. The radioactive methanol formed during this alkaline hydrolysis was extracted with 3 ml of toluene: isoamylalcohol (3:2 by vol.) and measured by subtracting the radioactivity left after evaporation of 1 ml aliquot from the total radioactivity present in a similar 1 ml aliquot.

Purification of protein carboxyl-methylase. Protein carboxyl-methylase was purified over 2000 fold from the cytosol of bovine erythrocytes using a method similar to that of Kim (20). The purification steps were: 1) a 55% ammonium sulfate precipitation of erythrocyte cytosol, 2) chromatography on Sephadex G-100, 3) a second 55% ammonium sulfate precipitation and, 4) chromatography on hydroxylapatite. The purified preparation was devoid of endogenous methyl acceptor proteins.

Distillation of radioactive methanol and formation of ^{3}H-methyl-3,5-dinitrobenzoate. The radioactive material originating from protein-^{3}H methyl esters and present in the trichloroacetic acid was distilled under reduced pressure using a microdistillation apparatus. The radioactive material was distilled at the same temperature as the cold methanol which was used as a carrier.

To ascertain that the radioactive material was radioactive methanol, the above distillate was reacted with 3,5-dinitrobenzoyl chloride and the derivative crystallized three times as described by Pasto and Johnson (21).

Protein determination. Protein concentrations were measured by the method of Lowry et al (22) using bovine serum albumin as a standard.

RESULTS

To investigate the presence of a protein methylesterase, radioactive protein-methyl esters were synthesized at pH 5.2 using gelatin as substrate, S-adenosyl-^{3}H methyl methionine and purified protein carboxyl-methylase. This low pH was chosen because of the low spontaneous hydrolysis of protein-methyl

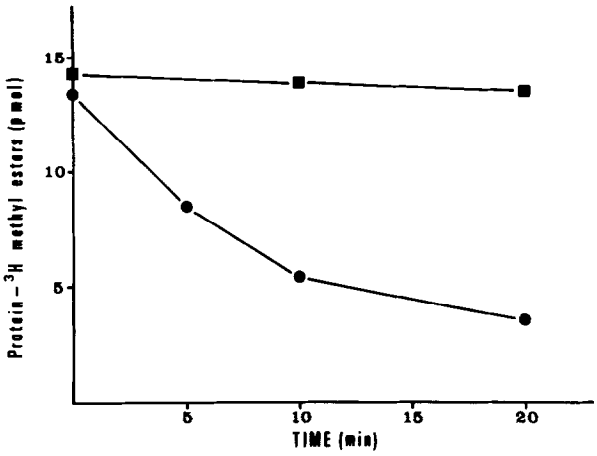

FIGURE 1. Hydrolysis of protein-^{3}H methyl esters by boiled and fresh kidney homogenate. Fresh homogenate (●——●) and 5 min. boiled homogenate (■——■).

esters at pH 5.2. Radioactive protein-methyl esters were then incubated with fresh or boiled kidney homogenate. In the presence of fresh kidney homogenate there was a progressive decrease of radioactive protein-methyl esters (Fig. 1). However in the presence of boiled kidney homogenate (Fig. 1) or 0.3 M sucrose (data not shown) only limited hydrolysis was observed (less than 7% after 20 min of incubation) representing spontaneous (non-enzymatic) degradation of protein-methyl esters.

To support the involvement of an enzyme in protein methyl ester degradation the effect of trypsin was investigated. A 10% kidney homogenate (W/V) was prepared in 0.3 M sucrose and centrifuged at 150,000 x g for 60 min. The high speed supernatant was treated with 0, 2 and 20 mg of trypsin for 2h. at 37^{o}C, then a five fold excess of trypsin inhibitor was added and protein-methylesterase activity determined. Trypsin treatment greatly diminished the capacity of kidney high speed supernatant to degrade radioactive protein-methyl esters (Table I). The disappearance of activity upon heating and proteolytic treatment indicates that an enzyme is responsible for hydrolysis of protein-methyl esters.

The radioactive product originating from the hydrolysis of protein-^{3}H methyl esters was investigated. This radioactive product was quantitatively

Table I. Effect of trypsin treatment on the capacity of kidney cytosol to hydrolyse protein-methyl esters.

Treatment	Protein-^{3}H methyl esters hydrolysed (pmol/assay)	% of inhibition
Cytosol + Trypsin (0 mg/ml)	6.1	0
Cytosol + Trypsin (2 mg/ml)	5.1	16
Cytosol + Trypsin (20 mg/ml)	1.0	84

Table II. Recovery of the product of hydrolysis of protein-^{3}H methyl esters as methanol*.

Time (min)	^{3}H-methyl in protein methyl esters (pmol)	^{3}H-methyl in methanol (pmol)	^{3}H-methyl total (pmol)	Recovery (%)
0	17.4	0.3	17.7	100
6	10.9	5.1	16.0	90
12	7.3	8.9	16.2	92

* Kidney cytosol was incubated for 0, 6 and 12 min with protein-^{3}H methyl esters. At the end of each period of incubation, 10% trichloroacetic acid was added and the ^{3}H methyl groups remaining in precipitated proteins were measured. The ^{3}H methyl in the trichloroacetic acid supernatant was distilled under reduced pressure using methanol as a carrier.

recovered under the same distillation conditions as cold methanol (Table II). A 3,5-dinitrobenzoate derivative was then made for further characterization and three crystallizations were performed. Constant melting point (105-107^{o}C) and constant specific activity (1320 CPM/mg) of ^{3}H methyl-3,5-dinitrobenzoate were reached after the second crystallization. The consistancy of specific activity upon repeated recrystallization together with the above results indicate that kidneys contain a protein methylesterase which hydrolyses protein-methyl esters to release methanol.

The level of protein methylesterase was investigated in various rat tissues. The enzyme activity was measured with and without 0.1% Nonidet-P-40, a non ionic detergent. Levels were always higher in the presence of detergent indicating that at least part of enzyme activity was membrane bound (Table III). The highest specific activities were found in kidney and liver.

The optimum pH for protein methylesterase was determined by incubating radioactive protein-methyl esters with fresh or boiled kidney cytosol. In the presence of boiled cytosol there was a spontaneous hydrolysis of protein-methyl

Table III. Levels of protein methylesterase in various rat tissue homogenates.

Tissues	Specific activity without detergent (pmol/min/mg prot.)	Specific activity with detergent (pmol/min/mg prot.)
Kidney	4.84	8.00
Liver	0.122	0.900
Adenohypophysis	0.088	0.359
Adrenal	0.082	0.593
Pancreas	0.181	0.357
Heart	0.042	0.143
Testes	0.124	0.550
Brain	0.031	0.170

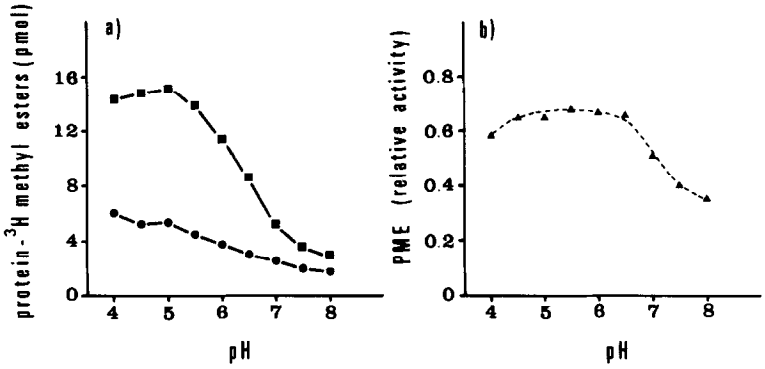

FIGURE 2. pH curve of hydrolysis of protein-^{3}H methyl esters by kidney cytosol. a) fresh (●——●) and boiled (■——■) kidney cytosol; b) protein methylesterase (PME) activity (▼- - -▼).

esters above pH 6.0 (Fig. 2a). However, in the presence of fresh cytosol, protein-methyl esters were hydrolysed at all pH values tested. From data on these two pH curves (Fig. 2a), a pH curve for protein methylesterase was generated by subtracting the values of the lower curve from that of the upper curve and dividing the difference by the values of the upper curve. The optimum pH was observed between 4.5 and 6.5 (Fig. 2b). At pH 8.0 the enzyme was still at 50% of its maximal activity.

DISCUSSION

In this paper, I have presented evidence for the existence of a mammalian protein methylesterase. This enzyme occurred in both the soluble and membrane bound fractions of several tissues. The highest tissue concentration was found in kidney. The enzyme had a broad range (pH 4.5 to 6.5) for optimal activity and at pH 8.0 retained half maximal activity.

Protein carboxyl-methylation has been associated with secretion. In adrenal medulla the best substrates for that enzyme were found on the chromaffin granule membranes (6,7). In posterior pituitary (8) and in parotid gland (11, 12) an increase in protein carboxyl-methylase activity was observed after stimulating secretion. An increase in in vivo protein carboxyl-methylation was also obtained after adrenal medulla stimulation (10).

In bacteria, the carboxyl-methylation of membrane proteins controls the direction of rotation of flagella and bacterial chemotaxis (13-16). Recently a protein methylesterase has been discovered in bacteria (23). This enzyme adds a second regulatory mechanism for chemotaxis. The relationship between protein methylation and chemotaxis seems to extend to mammals (leukocyte chemotaxis) (17, 18). Thus, the mammalian protein methylesterase described in this paper may well represent an important regulatory mechanism for secretion and leukocyte chemotaxis.

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